The porcine memory B cell in conferring long term adaptive immunity to viral pathogens

A Dissertation

SUBMITTED TO THE FACULTY OF

THE UNIVERSITY OF MINNESOTA

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Advised by *Dr. Michael P. Murtaugh*

June, 2017

Acknowledgements

I would like to thank my adviser, Dr. Michael P. Murtaugh, for his scientific guidance, encouragement, and the autonomy to pursue scientific questions and interests. Thank you to Dr. Cathy Carlson for her support as well as my thesis committee: Dr. Marc Jenkins, Dr. Kent Reed, and Dr. Carol Cardona for their mentorship.

I am extremely grateful to the members of the Murtaugh lab for sharing their knowledge, talents, and lives with me for the past four years. I am also thankful for my fellow graduate students. Sharing the adventure of graduate school with all of you has opened my eyes to diverse opinions and experiences which have provided me with a wealth of perspective.

My research was supported by NIH T32 Comparative medicine and pathology training grant, USDA-NIFA grant, Boehringer Ingelheim Vetmedica, Puretein biosciences and the Sam Maheswaran fellowship.

Finally, I'd like to acknowledge my family and friends. Each of you has inspired or motivated me to become the best version of myself in one way or another throughout my life but especially during my time in the twin cities. Thank you for all of your prayers and support.

Dedication

This dissertation is dedicated to my parents Dr. Dewayne and Krystal Rahe, who gave me my faith, instructed me in self-discipline, and have supported me throughout my educational endeavors.

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most important pathogen of swine health and wellbeing worldwide. Discovered nearly thirty years ago, there is still no vaccine capable of producing a broadly protective immune response against the virus. This deficiency is due in large part to a failure to understand how the adaptive immune system responds to vaccination or infection. Specifically, there is little to no knowledge regarding the all-important memory immune response to PRRSV. The objective of this dissertation was to fill in this significant gap in knowledge by identifying and characterizing the memory B cell response to PRRSV vaccination. First, we identified the presence of memory B cells against PRRSV non-structural protein 7 (nsp7) through the use of a novel *in vitro* B cell culture system. Next, we created and validated a novel reagent, a B cell tetramer, against nsp7 to enhance the speed and sensitivity of memory cell identification. Finally, through the utilization of the nsp7 tetramer, we evaluated the regional specificity and dynamic nsp7 specific B cell response to PRRSV MLV vaccination within select secondary lymphoid organs. These results constitute the first evidence of regional specialization of the B cell response to vaccination in an outbred animal species. Furthermore, the presented methods are a blueprint for the study of antigen specific cellular responses to any significant pathogen of animals important for food or fiber.

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List of Abbreviations

Ab	antibody
ADCC	antibody dependent cell-mediated cytotoxicity
AF680	alexa fluor 680
APC	allophycocyanin
APRIL	a proliferation inducing ligand
ASC	antibody secreting cell
ATP	adenosine triphosphate
BAFF	B cell activating factor
BCMA	B cell maturation antigen
BCR	B cell receptor
CDC	complement-dependent cytotoxicity
CFSE	carboxyfluorescein succinimidyl ester
DNA	deoxyribonucleic acid
ECAR	extracellular acidification rate
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
FACS	fluorescence-activated cellular sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FPKM	fragments per kilobase of transcript per million mapped reads
FSC	forward scatter
FVD	fixable viability dye
Gp	glycoprotein
HIV	human immunodeficiency virus
HP-PRRS	highly pathogenic porcine reproductive and respiratory syndrome
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin

IHC	immunohistochemistry
ILN	inguinal lymph node
KLH	keyhole limpet hemocyanin
mAb	monoclonal antibody
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MLV	modified live virus
mo-DC	monocyte-derived dendritic cell
mRNA	messenger ribonucleic acid
MSD	mystery swine disease
Mtb	*Mycoplasma tuberculosis*
NFDM	non-fat dry milk
NIH	National Institutes of Health
NK cell	natural killer cell
nsp	non-structural protein
OCR	oxygen consumption rate
OD450	optical density at 450 nm
ORF	open reading frame
pAb	polyclonal antibody
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween 20
PCR	polymerase chain reaction
PCV2	porcine circovirus
PE	phycoerythrin
PRRSV	porcine reproductive and respiratory syndrome virus
RNA	ribonucleic acid
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SA	streptavidin

SIRS	swine infertility and respiratory syndrome
SILN	subiliac lymph node
SLA	swine leukocyte antigen
SSC	side scatter
TACI	transmembrane activator and CAML interactor
TBLN	tracheobronchial lymph node
TGFβ-1	transforming growth factor beta - 1
Treg cell	T regulatory cell
USDA	United States Department of Agriculture

Chapter 1 - The adaptive immune response to porcine reproductive and respiratory syndrome virus

A manuscript submitted to Viruses:

Mechanisms of adaptive immunity to porcine reproductive and respiratory syndrome virus

Michael C. Rahe and Michael P. Murtaugh

1.1 Abstract

The adaptive immune response is necessary for the development of protective immunity against infectious diseases. PRRSV, a genetically heterogeneous and rapidly evolving RNA virus, is the most burdensome pathogen of swine health and well-being worldwide. Viral infection induces antigen-specific immunity that ultimately clears the infection. However, the resulting immune memory, induced by virulent or attenuated vaccine viruses, is inconsistently protective against diverse viral strains. The immunological mechanisms by which primary and memory protection are generated and used are not well understood. Here, we summarize current knowledge regarding cellular and humoral components of the adaptive immune response to PRRSV infection that mediate primary and memory immune protection against viruses.

1.2 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most severe enemy of porcine health and wellbeing. The highly mutable, enveloped, RNA virus was discovered nearly 30 years ago but, while extensive research has been carried out and many vaccines have been developed, there is still no reproducible immunological intervention that develops a broadly protective immune response against virulent PRRSV.

PRRS disease was first described on farms in North Carolina in the USA at the end of the 1980's. Outbreaks were marked by reproductive losses, post-weaning pneumonia, and increased mortality in growing pigs. Initial efforts to identify an etiological agent responsible for the new disease syndrome were unsuccessful, leading to the disease being temporarily designated mystery swine disease (MSD) in North America. Koch's postulates for MSD were fulfilled in 1991 with a previously unidentified RNA virus discovered in Europe, named Lelystad virus (Terpstra et al., 1991; Wensvoort et al., 1991). The discovery was quickly followed by isolation of the virus, initially referred to as swine infertility and respiratory syndrome virus or SIRS virus, in North America (Collins et al., 1992).

The name porcine reproductive and respiratory syndrome virus (PRRSV) was introduced in 1992 and now describes Type 1 (genotypes first isolated in Europe) and Type 2 (genotypes first isolated in North America) (Allende et al., 1999; Nelsen et al., 1999). Recent discovery of multiple PRRSV nucleotide sequences in nonhuman primates has led to a reclassification of PRRSV as PRRSV-1 and PRRSV-2 in place of type 1 and type 2, respectively (Kuhn et al., 2016). Today, both virus types are globally distributed, with

PRRSV-1 viruses predominantly in Europe and PRRSV-2 viruses largely in North America, Asia and South America (Zimmerman et al., 2012).

PRRSV is a positive-sense, single-stranded RNA virus of approximately 15 kb designated to the Arteriviridae family. The virus encodes at least ten functional open reading frames (ORF). ORF1a and 1b encode two large polyproteins which are cleaved into 14 non-structural proteins (Fang and Snijder, 2010). There are eight known structural proteins encoded by ORF2a, ORF2b, ORF3-7 and ORF5a (Dea et al., 2000; Johnson et al., 2011; Meulenberg et al., 1998). PRRSV is one of the most rapidly mutating RNA viruses known, with considerable genetic variation within both PRRSV-1 and PRRSV-2, based on ORF5 phylogenetic analysis (Brar et al., 2015; Murtaugh et al., 2010). This impressive genetic diversity makes the development of a broadly protective immune response to vaccination difficult to achieve. After infection, the virus can persist and replicate in the host, depending on immune status and PRRSV strain, for a period of at least 150 days (Allende et al., 2000). Therefore, contrary to being labeled repeatedly as a persistent pathogen, animals are capable of eventually clearing PRRSV. However, the components of the immune system responsible for the development of sterilizing immunity are not completely understood or have yet to be discovered. Here, we will discuss several aspects of PRRSV antigen-specific and protective immunity which have yet to be elucidated while focusing on potential areas of further investigation. Readers interested in additional reviews of PRRSV literature related to immunity are directed to the following articles (Loving et al., 2015; Lunney et al., 2016).

1.3 The targets of infection

PRRSV infects cells of the macrophage/monocyte lineage, including dendritic cells (Duan et al., 1997b; Kim et al., 1993; Lawson et al., 1997; Park et al., 2008). Permissive cells express CD163, a hemoglobin-haptoglobin scavenger, which is the necessary receptor for PRRSV infection and replication (Gao et al., 2013; Prather et al., 2017; Whitworth et al., 2016). Macrophages and dendritic cells play a varied, and important, role in many aspects of, tissue remodeling, development, immunity and immunopathology. Classically designated as part of the innate immune system, these leukocytes are critical for the development of a productive adaptive immune response. Macrophages and, particularly, dendritic cells take up and present antigen to T cells and B cells, thus initiating an adaptive immune response against the presented antigen (Batista and Harwood, 2009; Katz and Unanue, 1973). If a pathogen is able to infect and destroy, manipulate, or maintain itself within macrophages or dendritic cells, it then has the potential to modulate the immune response into a favorable situation for its own replication and survival.

Therefore, many pathogens employ strategies for macrophage infection as a way to make the host more amenable to infection. Recent research into *Mycobacterium tuberculosis* (Mtb) has shown that, after phagocytosis, the bacterium arrests phagosome maturation and intra-phagosome lipolysis resulting in Mtb survival and an increased supply of nutrients for growth (Kalam et al., 2017; Podinovskaia et al., 2013). Human immunodeficiency virus (HIV) infects macrophages to establish reservoirs within the host for the chronic stage of the disease when $CD4^+$ T cells are largely depleted and neutralizing antibodies may be present (Abbas et al., 2015; Koppensteiner et al., 2012a;

Koppensteiner et al., 2012b). *Leishmania major* is a protozoan which infects phagocytes to subvert the immune system. The parasite expresses gp63, a multifaceted surface-expressed pathogenicity factor that is responsible for preventing antigen presentation and killing by NK cells (Ghosh et al., 2013; Matheoud et al., 2013; Moradin and Descoteaux, 2012). Indeed, there are many more examples of burdensome pathogens which target phagocytic cells, especially macrophages and dendritic cells, in an attempt to gain a foothold within the immune system and allow for unchecked survival and replication (Laksono et al., 2016; Lam et al., 2012; Lee and Lee, 2015). PRRSV is one of these pathogens.

The ability of PRRSV to subvert the immune system has not been investigated as extensively as more prominent pathogens of humans, such as HIV. PRRSV has been shown to inhibit the production, or the downstream effects, of type 1 interferons, particularly interferon-α, on intracellular signaling (Albina et al., 1998; Beura et al., 2010; Huang et al., 2014; Sun et al., 2016; Sun et al., 2012b; Yang et al., 2016; Yang and Zhang, 2016). Interestingly, multiple PRRSV proteins (nsp1, nsp2, nsp4, nsp11 and N) have been reported to possess interferon inhibiting abilities.

In addition, a number of *in vivo* experiments have reproduced earlier *in vitro* findings showing that interferon-α is inhibited during the early stages of PRRSV infection (Albina et al., 1998; Buddaert et al., 1998; Van Reeth et al., 1999). While the impact of type 1 interferon suppression is likely to create a favorable environment for the virus to replicate and survive in phagocytic cells, it is still unclear what effect, if any, suppression of type 1 interferon activity has on the adaptive immune response to infection (Mulupuri et al., 2008). Future investigations could clarify the relative contributions of viral proteins on

modulation of interferon production and their impacts on viral growth, survival, and the subsequent development of the adaptive immune response.

Apart from interfering with interferon expression, PRRSV has also displayed the *in vitro* ability to subvert the immune system by spreading from cell to cell. Recent work has uncovered the ability of the virus to spread infectious viral RNA, several replicases, and certain structural proteins between cells via intercellular nanotubules (Guo et al., 2016). While this activity theoretically allows for PRRSV to avoid neutralizing antibodies, the presence and significance of this mechanism in PRRSV pathogenesis has yet to be fully elucidated. Future studies are needed to determine if this process operates in the naturally permissive macrophage and dendritic cell, if it can be interrupted, for example by intracellular antibodies, and what effect it might have on viral propagation (Bai et al., 2011; Corthésy et al., 2006).

Vaccines depend upon innate immune stimulation to promote effective adaptive immune response to antigen, resulting in production of antibodies and cytotoxic T cell responses. The ability of a pathogen to successfully infect and replicate within innate immune cells makes the development of a protective immune response more difficult. As a result, the generation of effective vaccines against pathogens which target immune cells is fraught with challenges. Extensive variation in viral genetics, in primary immune responses, and in cross-protection indicates that much remains to be learned about cellular pathogenesis in order to arrive at better immunological solutions.

1.4 PRRSV immunosuppression and adaptive immunity

Immunosuppression refers to suppression of the immune system and its ability to fight infection. HIV and infectious bursal disease virus are examples of viral infections that destroy entire lymphoid cell populations that ablate or disable adaptive immune responses. Lymphoproliferative cancers block cellular differentiation and deprive the body of mature, effector lymphocytes, thus causing immunosuppression in a different manner. PRRSV does neither; infection does not lead to severe lymphoid depletion or ablation, and it does not interfere profoundly with lymphocyte differentiation or maturation. Leukocyte perturbations in lymphoid tissues is associated with PRRSV infection, suggesting that adaptive immunity might be weakened, though not destroyed (Amarilla et al., 2016; Cao et al., 2013; Gómez-Laguna et al., 2013; Li et al., 2014b; Sinkora et al., 2014; Zhang et al., 2016).

The immune system also maintains peripheral tolerance to self and commensal bacteria through immunosuppressive mechanisms that include regulatory T cells (Tregs), characterized as CD4+CD25+Foxp3+ T lymphocytes (Pandiyan et al., 2011). Treg suppressive properties were discovered when thymectomized or Treg-depleted mice succumbed to autoimmune reactions (Sakaguchi et al., 1982; Sakaguchi et al., 1996). Tregs suppress effector and effector memory T cell proliferation by cytokine deprivation leading to polyclonal apoptosis, and by suppression of antigen presenting cells by CTLA-4 and other mechanisms (Pandiyan et al., 2011). Studies in PRRSV infections give an ambiguous picture about the role of Tregs. PRRSV-2 strains are reported to induce a strong Treg response which included TGFβ-1 secretion *in vitro* as well as *in vivo* (Silva-Campa et al., 2009; Wongyanin et al., 2010). Other studies did not show Treg responses

to infection with either PRRSV-1 or PRRSV-2 (Rodríguez-Gómez et al., 2015; Silva-Campa et al., 2010). Interleukin-10 (IL-10), an immunosuppressive cytokine expressed by various cell types including Tregs, was induced by PRRSV-2 vaccination in weaned pigs in one study, but was not induced in weaned or adult pigs in another study (Klinge et al., 2009). Additional *in vitro* and *in vivo* studies reported IL-10 mRNA transcription and cytokine production after PRRSV infection (García-Nicolás et al., 2014; Gómez-Laguna et al., 2009; Suradhat and Thanawongnuwech, 2003). However, kinetic analysis in sera of viremic pigs of various ages showed that elevated IL-10 levels were primarily a function of age and were not associated with infection status (Klinge et al., 2009). The only exception was in weaned pigs infected with a virulent virus, in which a transient increase was associated with viral pathogenesis (Klinge et al., 2009).

On balance, the immunological evidence for PRRSV inducing a state of immunosuppression does not appear to be compelling. Secondary infections following PRRS outbreak in swine herds, suggesting a reduced ability to fight infection, is an alternative indicator of immunosuppression. An early study showed concurrent pulmonary bacterial infections in 58% of 221 PRRS cases (Zeman, 1996). However, the study did not determine if bacterial infections were present before the PRRS outbreaks. The immunosuppression question also was addressed in more controlled settings using dual infection models with PRRSV and various bacterial species. A summary of published literature in 2003 showed no predisposition to bacterial disease in 8 of 15 coinfection models, 3 ambiguous outcomes, and 4 cases in which severity of disease was increased (Halbur, 2003). More recent studies found a positive association between

PRRSV infection and replication of porcine circovirus 2 (PCV2) or swine influenza virus (Dobrescu et al., 2014; Sinha et al., 2011).

It is possible that bacterial infections in swine herds increase following PRRS outbreaks due an increased burden of viral infection on host resilience to pathogen burden. Subclinical viral and bacterial infections are common, with PCV2, *Salmonella enterica*, *Haemophilus parasuis*, various *Mycoplasma* species, Leptospira, and *Escherichia coli* being examples. Control of infection is maintained by a combination of immune resistance to microbial replication and tissue tolerance to damage. In a coinfection model of influenza virus and *Legionella pneumophila*, it was clearly demonstrated that *L. pneumophila* infection was subclinical in healthy mice, but was lethal in the presence of influenza virus (Jamieson et al., 2013). Overwhelming disease was due to loss of tissue resilience, since the bacterial load was unchanged (Jamieson et al., 2013). This model might account for mortalities observed in experimental swine following PRRSV exposure (Johnson et al., 2004). Given the variable results of PRRSV coinfection models in swine and an alternative mechanism for increased disease in PRRSV-infected herds, generalized immunosuppression does not appear to be a key feature of PRRSV pathogenesis.

PRRSV, like many viruses, has developed countermeasures to host immune responses that enable it to survive and replicate for extended periods of time before the infection is resolved. PRRSV modulation of intracellular antiviral defense mechanisms has been reviewed extensively (Butler et al., 2014). The effects of PRRSV infection on adaptive immune response, i.e. antigen-specific T cell, B cell, and antibody responses, are less well characterized. The antiviral response of T cells to PRRSV, examined primarily by

the IFNγ ELISPOT, appears to develop slowly over a period of weeks, and is not associated with changes in viral loads in blood or in infected lung and lymphoid tissues (Brown et al., 2009; Xiao et al., 2004). Peripheral blood mononuclear cells (PBMC) from young, weaned pigs show limited IFNγ responses even when stimulated by phytohemagluttinin, which might account for the low anti-PRRSV responsiveness after re-stimulation *in vitro* (Klinge et al., 2009). However, PBMC from growing pigs and mature sows, which showed higher levels of IFNγ responsiveness, still showed limited responsiveness (Klinge et al., 2009). These findings indicate that PRRSV may interfere with specific cell-mediated immunity, but more direct evidence is needed for a fuller understanding.

By contrast, the interaction of PRRSV with pigs does not appear to retard or attenuate the development of humoral immunity or B cell differentiation. Induction of antibody responses to PRRSV proteins, both structural and non-structural, occurred in the same time frame as antibody responses to keyhole limpet hemocyanin (KLH), an irrelevant protein antigen (Mulupuri et al., 2008). The antibody response to KLH also was the same in the presence or absence of PRRSV infection (Mulupuri et al., 2008). Thus, the adaptive B cell response is not delayed or suppressed by PRRSV.

An extended viremia and prolonged survival in lymphoid tissues is characteristic of PRRSV infection. These features show that PRRSV has mechanisms of immune avoidance that are not present in viruses such as influenza virus and foot and mouth disease virus, in which sterilizing immunity is achieved within 10 to 14 days. It appears from the findings of field observations and experimental investigations that some type of

PRRSV-specific T cell interference is present, whereas specific B cell inhibition or a generalized state of immunosuppression are not immunological hallmarks.

1.5 Antibody response

Neutralizing antibody response

The antibody response to PRRSV typically dominates discussions of PRRSV immunity, as neutralizing antibodies are the crucial component of immune-mediated protection against most viral infections (Burton, 2002; Plotkin, 2010). As a result, shortly after the identification of PRRSV as the causative agent of MSD there was a strong push to identify the presence and dynamic response of neutralizing antibodies against PRRSV and then to characterize their specificity for PRRSV variants. Early work suggested that neutralizing antibodies against homologous PRRSV could be found as early as 9-11 days after inoculation (Yoon et al., 1994). However, this was likely the non-affinity matured IgM response, as anti-swine IgM ablated the previously observed neutralizing activity. Subsequent research showed that the high affinity neutralizing IgG response, detected at around 28-42 days post-inoculation, is specific for the inoculating virus with only some neutralizing activity against heterologous virus (Bilodeau et al., 1994; Correas et al., 2017; Labarque et al., 2000; Li et al., 2014a; Loemba et al., 1996).

Following the identification of PRRSV neutralizing antibodies, the effectiveness of immunoglobulins in protecting against infection was evaluated with passive transfer studies. These experiments displayed the effectiveness of neutralizing antibodies at preventing clinical infection and disease against homologous challenge (Lopez et al., 2007; Osorio et al., 2002). However, these studies also showed that immune protection

can be quite limited, conferring protection against homologous inoculation with only partial protection against heterologous challenge (Choi et al., 2016; Lager et al., 1999; Wang et al., 2016). These results appeared to explain the potential field problem, in which vaccinated or live virus inoculated animals became infected with a variant PRRSV genetically different enough from the inoculating strain to evade the immune system, propagate, and then cause disease. Hence, ever since the mutability, antigenic variability, and resultant immunological elusiveness of PRRSV were first appreciated, a broadly neutralizing antibody response to PRRSV has been coveted by immunologists and practitioners (Dea et al., 1996).

Recent research shows that there are animals capable of developing a broadly neutralizing antibody response to genetically disparate viruses (Robinson et al., 2015; Trible et al., 2015). However, this immune capability has only been found in a proportion of animals in groups of similar genetics age, sex, and exposure history (Robinson et al., 2015). The seemingly random ability of some animals to develop broadly neutralizing antibodies suggests that the inherent variation of the adaptive immune response may play a role in conferring broadly neutralizing capabilities to certain animals. Investigations into this ability are needed at the lymphocyte level and while the obvious target is the B cell, T cells cannot be overlooked, as the induction of a humoral immune response requires antigen-specific T cell driven help (Schimpl and Wecker, 1973; Singer and Hodes, 1983). Therefore, animals able to develop a strong neutralizing antibody response would require both B cells and T cells that are capable of recognizing neutralizing epitopes. In any case, the conditions and interventions necessary to produce a broadly

neutralizing antibody response or to expand it to every animal within a herd are still unclear.

Recently, vaccinology research in HIV has shown that sequential immunizations, tailored for specific stages of the immune response, may be useful for inducing broadly neutralizing antibodies (Dosenovic et al., 2015; Jardine et al., 2016; Sanders et al., 2015). The approach is based on the finding that early immune responses to HIV resulted in neutralizing antibodies against the circulating virus which quickly led to immune escape of the virus and the ineffectiveness of generated antibodies. The antibody-resistant virus then stimulated a secondary antibody response which again selected for antibody resistant virus. This virus-antibody hide and seek continued, eventually resulting in the selection of several neutralization targets of the virus as well as the generation of broadly neutralizing antibodies (Bonsignori et al., 2011; Bonsignori et al., 2016; MacLeod et al., 2016). Cloning of the antibodies showed that somatic mutations are generally necessary for neutralizing capabilities of HIV-1 (Jardine et al., 2013; Scheid et al., 2009). These findings have shown that the B cell response of the host adapts in the germinal center as the virus evolves, suggesting that tailored sequential immunization could lead to the development of a broadly neutralizing antibody response (Escolano et al., 2017).

The consistent generation of a broadly neutralizing antibody response to PRRSV on the herd level has evaded the swine health industry since the emergence of PRRSV. There are multiple proposed mechanisms by which PRRSV may evade or inhibit the development, or the effectiveness, of a neutralizing antibody response, such as glycan shielding of GP3 or GP5 (Ansari et al., 2006; Vu et al., 2011), the existence of decoy epitopes in GP5 (Ostrowski et al., 2002), lymphocyte dysregulation (Butler et al., 2014),

and inhibition of the innate immune response (Sang et al., 2011). Comprehension of defense mechanisms employed by PRRSV makes the development of a broadly neutralizing immune response appear to be a daunting task. However, as previously shown, some animals are capable of developing such a response. Simply, the key to adapting the immune phenomenon of some animals to a vaccine capable of inducing broadly protective immunity in many animals lies in identifying conserved epitopes on surface proteins which are necessary for infection.

While the purported targets of neutralization have been extensively discussed in recent reviews, it is worth noting that several epitopes on the M protein, GP5, GP2, GP3, and GP4 have been shown, or implicated, to harbor neutralizing activity (Cancel-Tirado et al., 2004; Costers et al., 2010; Das et al., 2010; Delputte et al., 2004; Ostrowski et al., 2002; Plagemann et al., 2002; Vanhee et al., 2011; Vu et al., 2011; Zhou et al., 2012). However, knocking out only CD163 in the pig is sufficient to render animals non-susceptible to PRRSV infection and replication (Burkard et al., 2017; Prather et al., 2017; Whitworth et al., 2016). It is proposed that following endocytosis, CD163 associates with the virus within the endosome, resulting in uncoating of the virus and the release of the viral genome into the cellular cytoplasm (Van Breedam et al., 2010). Since CD163 is necessary for viral infection and replication, the logical next step is to identify the conserved regions of viral surface proteins, most likely the minor glycoproteins (GP2, GP3, and GP4), that interact with CD163.

Non-neutralizing antibody response

Traditionally, the non-neutralizing antibody response to PRRSV has been considered useful only for its ability to identify if an animal had been exposed and seroconverted to virus. Indeed, there are many structural and non-structural proteins of PRRSV which make this possible through their ability to induce a robust humoral immune response (Brown et al., 2009; Johnson et al., 2011; Molina et al., 2008). However, recent research on other pathogens has shown that non-neutralizing antibodies may play a much larger role in immunity than was previously appreciated (Ackerman and Alter, 2013; Long et al., 2017; von Bredow et al., 2016; Wong et al., 2017). Alternative antibody functions, such as antibody dependent cell-mediated cytotoxicity (ADCC), antibody-dependent complement-mediated cytotoxicity (CDC), and antibody-dependent complement-mediated virolysis may be important in the clearance of virus and virally infected cells from an animal. To our knowledge, there are only two published papers investigating non-neutralizing antibody functions in the context of PRRSV infection (Cao et al., 2013; Costers et al., 2006). Both of these *in vitro* studies utilized a PRRSV-1 virus and failed to find an effect of ADCC and CDC on infected cells. However, experiments focused on PRRSV-2 viruses with extended time points beyond 12 h are warranted. A more extensive review of non-neutralizing antibody functions can be found in the cited review (Rahe and Murtaugh, 2017a).

1.6 The B cell response

If antibodies are the most important effectors of the immune system against viral infection, then B cells that make the antibodies are the most important cells. Previous research on the interaction between PRRSV and the porcine B cell is contradictory. It has recently been suggested that PRRSV infection results in lymphocyte apoptosis and immune impairment (Gómez-Laguna et al., 2013). Several sources have shown that PRRSV largely or exclusively induces a specific humoral response to infection (Lamontagne et al., 2001; Mulupuri et al., 2008). Other studies report that PRRSV infection results primarily in polyclonal B cell activation leading to hypergammaglobulinemia and the development of immune complexes (Butler et al., 2008; Lemke et al., 2004; Plagemann et al., 2005; Sun et al., 2012a). The majority of work describing infection leading to polyclonal activation and hypergammaglobulinemia was performed in germ-free isolator piglets. This model is very effective for comparing B cell and antibody repertoire development in the fetus, as the germ-free status of the pigs removes many of the variables present when experiments are performed on conventionally reared animals (Butler et al., 2001). However, these animals are deprived of the microflora and maternal antibodies to which conventional animals are exposed. As a result, the translation of immunological outcomes observed in isolator pigs to conventional pigs must be performed with caution. Studies in mice show that the immune systems of specific-pathogen free laboratory mice are similar to neonatal human immune systems, whereas feral mice displayed immune systems more comparable to adult humans. Effectively, the immune systems of germ-free animals may not display “normal”

immune system phenotypes due to the lack of exposure to microflora (Beura et al., 2016; Graham, 2016).

The development of protective humoral immunity, after vaccination or exposure to a pathogen, is dependent upon two lines of defense. The first immune defense is secreted antibodies, first from short-lived and then from long-lived, plasma cells residing somewhere in the body (Fig. 1). The second line of defense is memory B cells (Fig. 1). Memory cells are sentinels against reinfection which are activated upon antigen recognition to proliferate and differentiate into antibody secreting plasma cells, thus rapidly boosting circulating antibody titers with high affinity class switched antibodies (Ahmed and Gray, 1996).

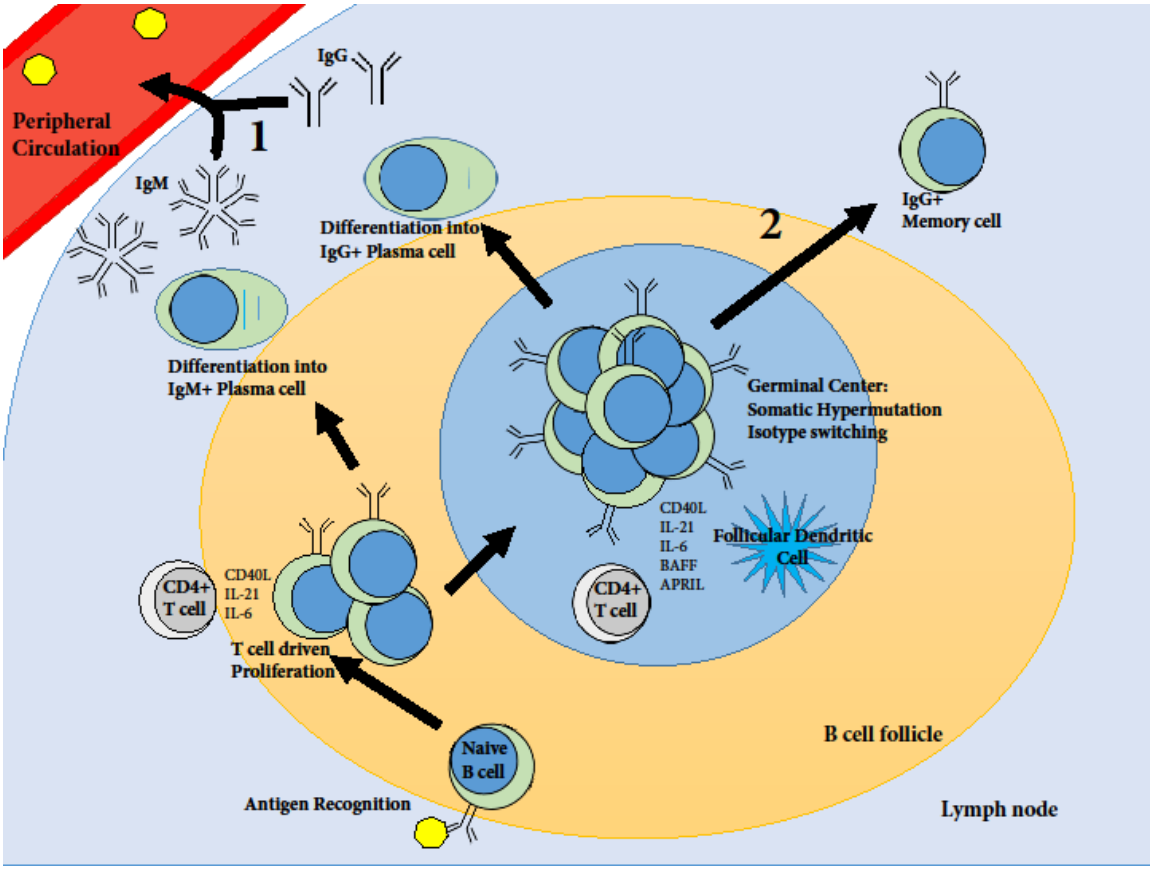

Figure 1-1 Development of systemic humoral immunity.

Figure 1 - Development of systemic humoral immunity. Naive B cells move through the B cell follicles of the secondary lymphoid organs searching for antigens specific for their B cell receptors (BCR, surface immunoglobulin). Upon antigen recognition, the BCR is endocytosed, the antigen is degraded and then presented on the surface of the cell via MHCII. The B cell then migrates to the periphery of the B cell follicle searching for a CD4+ T cell specific for the same antigen. Upon T cell recognition of the MHCII presented antigen, the T cell stimulates the B cell by cytokine driven proliferation. The B cell proliferates and differentiates, some cells become IgM producing plasma cells, and other cells migrate into the B cell follicle where, with the help of cytokines from CD4+ follicular helper T cells and follicular dendritic cells, a germinal center is formed. In the germinal center, B cells proliferate and undergo somatic hypermutation and isotype switching. Affinity matured B cells then leave the germinal center as either IgG+ plasma cells or IgG+ memory cells. These cells constitute the first two lines of defense against reinfection; (1) Affinity matured antibodies produced by plasma cells, and (2) Memory cells which boost antibody titers upon antigen recognition. For an in depth review of this process based on data in humans and mice, please refer to Taylor et al. (Taylor et al., 2012a).

Currently, there is scant research on the memory B cell response to PRRSV. Strong memory responses have been shown against nsp2, nsp7, N, and the 3' end of GP5 (Mulupuri et al., 2008; Rahe and Murtaugh, 2017b). The specific memory B cells are abundant in tonsil, lymph nodes draining the lungs and reproductive tract, and spleen. Unfortunately, there are many questions about the porcine memory response to PRRSV which have yet to be answered, including if memory cell kinetics closely mimic antibody kinetics, the response of PRRSV-specific memory pools upon homologous or heterologous viral challenge, and the importance of these cells in conferring protection against challenge. The development of sensitive and specific reagents, such as B cell tetramers, is a first step in being able to answer these critical questions. Additionally, it is possible that the key to understanding the broadly neutralizing response to PRRSV lies within circulating or lymphoid organ resident memory B cells. The potential to investigate these cells for identification of heavy and light chain antibody sequences is reviewed in Rahe and Murtaugh (Rahe and Murtaugh, 2017a).

Plasma cells

Plasma cells are terminally differentiated B cells responsible for making antibodies. Apart from the immature plasmablast, two types of plasma cells have been defined in the mouse and human (Radbruch et al., 2006; Slifka et al., 1998). Short-lived plasma cells quickly boost antibody titers while long-lived plasma cells maintain circulating antibody titers in the face of continual antibody degradation. Mulupuri et al. identified PRRSV-specific plasma cells in several secondary lymphoid organs, such as the spleen, tonsil, sternal lymph node, and inguinal lymph node (Mulupuri et al., 2008). Interestingly, no

PRRSV or irrelevant antigen (KLH)-specific plasma cells were found in the bone marrow (Mulupuri et al., 2008). This was surprising, as the bone marrow has been long considered as the reservoir for long-lived plasma cells in both mice and humans (Benner et al., 1974; McMillan et al., 1972; Slifka et al., 1995). It then begs the question, do pigs have long-lived plasma cells and if so, where do they reside? Mulupuri et al. found PRRSV and KLH specific plasma cells in secondary lymphoid organs 120 days after inoculation (Mulupuri et al., 2008). However, these cells may not be "long lived" as the prolonged viremia of PRRSV may result in a somewhat continuous stimulation of memory B cells resulting in the appearance of this plasma cell population in secondary lymphoid organs.

It seems unlikely that pigs do not have long lived plasma cells, as the half-life of porcine antibodies in serum is, on average, approximately 9 days (Curtis and Bourne, 1973; Polo et al., 2012). Therefore, without long lived plasma cells, pigs would quickly lose humoral protection as antibody titers waned. The identification of the anatomic location as well as the understanding of mechanisms for inducing a strong long lived plasma cell response may be important for future vaccine design as well as comprehending host-pathogen interactions.

1.7 T cell response

Interestingly, even though neutralizing antibodies have historically garnered the majority of attention in PRRSV immunology, it is well-known that pigs readily control infection in the absence of neutralizing antibodies. Furthermore, viremia is reported in the presence of neutralizing antibodies (Batista et al., 2004; Fontanella et al., 2017). Therefore, there

must be other facets of the immune system which effectively function to control infection and eliminate PRRSV from the host. While some of this activity may be attributed to non-neutralizing functions of antibodies, the T cell response to infection demands further investigation. A recent PRRS immunity review summarized previous research on functional T cell subsets, and PRRSV epitope targets, as well as gaps in T cell immunity (Loving et al., 2015). Here, we provide context for the understanding of novel results which have not been comprehensively reviewed, as well as reviewing foundational advances in the T cell response to PRRSV.

Early research focused on describing differences in T cell phenotypes post-PRRSV infection identified a large, transient decrease in the $CD4^+/CD8^+$ T cell ratio early, usually within the first week, in the course of infection (Shimizu et al., 1996). The change in this ratio could have been due to a temporary loss of $CD4^+$ cells through apoptosis or to an increase in $CD8^+$ cells due to antigen-specific proliferation (Kawashima et al., 1999; Shimizu et al., 1996). The importance of these findings for clearance of PRRSV or protection from infection were not known at the time, and other explanations, such as fluxes in cell populations between spleen, other lymphoid tissues, and blood could not be discounted. Furthermore, other studies have reported no significant change in $CD4^+$ or $CD8^+$ T cell frequencies in infected animals during the acute stage of infection (Silva-Campa et al., 2012; Xiao et al., 2004). In addition, Xiao et al. study showed that the abundance of virus-specific T cells in secondary lymphoid tissues did not correlate with viral load within the same tissue at either 19 or 67 days post-infection (Xiao et al., 2004). While this may suggest that T cells don't play a role in the elimination of virus, this study was limited by only two time points. Therefore, it's

difficult to interpret the significance of the presence, or absence, of PRRSV-specific T cells and the absence of virus by correlation alone.

Subsequent work reported that Foxp3+ Tregs were induced after PRRSV-2 infection (Silva-Campa et al., 2009; Silva-Campa et al., 2012). Unfortunately, these studies failed to investigate the development of other components of the adaptive immune response to PRRSV by comparing to a strain which is known to not induce a strong T-reg response (Silva-Campa et al., 2010). Therefore, the significance of these findings for the generation of a productive adaptive immune response is difficult to determine. However, future investigation is warranted.

Experiments to solve the Th1/Th2 paradigm in the pig showed that PRRSV induced a strong Th1 response, as expected, identified *in vivo* by an increased expression of T-bet in $CD4^+$ cells (Ebner et al., 2014). However, the finding is at odds with previous reports indicating that PRRSV infection results in the production of IL-10, a cytokine classically associated with a Th2 phenotype. Similarly, monocyte-derived dendritic cells (Mo-DCs) infected with PRRSV down regulate SLA-I, SLA-II, CD40 and CD80 as well as promote IL-10 secretion over IL-12 secretion (Liu et al., 2016). Delineation of the Th1/Th2 response to PRRSV, elucidation of Th1/Th2-specific cytokine markers in swine, as well as identifying associated cytokine responses of dendritic cells within secondary lymphoid organs where T cell proliferation and differentiation is most likely to occur, would help to resolve these outstanding questions (Murtaugh et al., 2009).

The Th17 cell has classically been identified, in mouse and human, as playing an important role in extracellular bacterial immunity through the production of the pro-inflammatory cytokines, IL-17A, IL-17F, and IL-22 (Kudva et al., 2011; Liang et al.,

2006). IL-17 producing Th17 cells are known to exist in the pig (Pilon et al., 2009). The importance of this T cell subset in the context of PRRSV infection has recently been investigated. A strain of Chinese highly pathogenic PRRSV (HP-PRRSV) appeared to suppress Th17 cells in the peripheral blood and lungs of pigs, resulting in an increased susceptibility to secondary bacterial infections (Zhang et al., 2016). Remarkably, the effect was PRRSV strain-specific, as a non-HP PRRSV strain failed to elicit the same response. Future research into the T cell response to PRRSV, especially with T cell tetramers and functional ELISPOTs, will be essential for the characterization of both $CD4^+$ and $CD8^+$ antigen specific T cells. Understanding how antigen-specific T cells interact with both infected and uninfected antigen presenting macrophages and dendritic cells will be helpful for advancing the field of PRRSV immunity.

1.8 Natural killer cell (NK) response

The natural killer cell is an innate lymphoid cell which can have a profound impact on adaptive immunity, but is also able to induce an early and rapid innate response against pathogens through a variety of mechanisms. NK cells produce cytokines, such as IFN-γ, are cytotoxic activity against infected cells not expressing MHC-I, can induce dendritic cell maturation, and effect the destruction of infected cells in ADCC (Shekhar and Yang, 2015). However, NK cells may deploy even more extensive and important functions in porcine immunity than are currently realized.

An early clue that NK cells were involved in innate responses to PRRSV was a sharp peak in serum IFN-γ shortly after infection (Wesley et al., 2006). The acute response was attributed to NK cells, as the result was deemed too early for a T cell response, and

suggested that decreased viral burdens in the lung prior to humoral or T cell responses could be due to the function of NK cells. However, it is known that porcine macrophages are also capable of producing IFN-γ in the presence of PRRSV infection (Choi et al., 2002; Thanawongnuwech et al., 2003). Furthermore, PRRSV appears to suppress the NK cell response without significantly affecting NK cell numbers (Dwivedi et al., 2012; Jung et al., 2009; Manickam et al., 2013; Renukaradhya et al., 2010). The cause of this suppression has yet to be determined, although viral proteins, rather than soluble factors from cells, may be responsible (Cao et al., 2013). Potential roles of additional NK cell functions, such as ADCC, in PRRSV immunity are poorly understood (Rahe and Murtaugh, 2017a).

1.9 Conclusion

PRRSV has tormented the health and wellbeing of swine worldwide since its discovery in the late 1980's. Unfortunately, after almost 30 years of research into the porcine immune response to PRRSV, there is still no effective means for inducing a broadly protective immune response at the herd level. The reasons for this failure are not completely known, but presumably include mechanisms by which the virus subverts the immune system. The ability of the virus to rapidly mutate while not losing fitness challenges the host immune system to keep pace. At the same time, infection of macrophages, a key player in immunoregulation, challenges both innate and adaptive immune cell mobilization as well as induction of a coordinated response that is needed for effective control and elimination of the virus.

Fortunately, foundational advances in the understanding of viral pathogenesis and immunity are enabling more informative investigations. The identification of CD163 as the necessary and sufficient receptor for infection supports the implications of broadly neutralizing antibodies that a conserved target is present on all PRRSV. Understanding how PRRSV surface glycoproteins interact with CD163 should lead to the identification of conserved epitopes which are necessary for infection. If, as appears to be the case, there is only one conserved way into the cell, then there must be a conserved viral sequence, or structure, which enables viral entry. Furthermore, the knowledge that pigs eventually develop sterilizing immunity, if given enough time, supports the concept that conserved epitopes exist on the virus. Therefore, the study of mature animals, which have cleared the virus, may provide the key to understanding how the immune system eventually gets the upper hand on the virus and cures infection.

Even with seminal advances in several aspects of the study of PRRSV, there remains much to be understood and clarified. Currently, the published literature presents conflicting views on many aspects of PRRSV adaptive immunity, especially related to T and B cell responses and the production, or inhibition, of cytokines in the face of infection. The continued development of antigen-specific reagents, of high sensitivity and specificity, is needed for understanding how the host responds to PRRSV infection. Furthermore, it is important that future PRRSV studies focus on the relevant host animal, the conventional pig. While the study of this outbred animal species is perhaps challenging at times, it affords the ability to study the host-pathogen interaction in the only species in which the virus naturally interacts. Additionally, knowledge gained about

the immunology of conventional pigs will accelerate immunological elucidation of other pig-pathogen interactions.

In conclusion, PRRSV continues to be the most burdensome pathogen of pigs worldwide, due to its propensity for immune evasion and manipulation. However, the continued study of the porcine immune response to infection, with improved reagents and methods, will illuminate those aspects of the host-pathogen interaction which are now hidden. It is through these discoveries that the complex question that is PRRSV will finally be answered.

1.10 Acknowledgements

The authors were supported in part by Agriculture and Food Research Initiative Competitive Grant no. 2016-67015-24928 from the USDA National Institute of Food and Agriculture and NIH award T32 OD010993 for support of MCR.

Chapter 2 - Interleukin-21 drives proliferation and differentiation of porcine memory B cells specific to PRRSV nsp7 into antibody secreting cells

A manuscript published in PLoS One 12 (2017): e0171171

Interleukin-21 drives proliferation and differentiation of porcine memory B cells into antibody secreting cells

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2.1 Abstract

Immunological prevention of infectious disease, especially viral, is based on antigen-specific long-lived memory B cells. To test for cellular proliferation and differentiation factors in swine, an outbred model for humans, $CD21^+$ B cells were activated *in vitro* with CD40L and stimulated with purported stimulatory cytokines to characterize functional responses. IL-21 induced a 3-fold expansion in total cell numbers with roughly 15% of all B cells differentiating to IgM or IgG antibody secreting cells (ASCs.) However, even with robust proliferation, cellular viability rapidly deteriorated. Therefore, a proliferation inducing ligand (APRIL) and B cell activating factor (BAFF) were evaluated as survival and maintenance factors. BAFF was effective at enhancing the viability of mature B cells as well as ASCs, while APRIL was only effective for ASCs. Both cytokines increased approximately two-fold the amount of IgM and IgG which was secreted by IL-21 differentiated ASCs. Mature B cells from porcine reproductive and respiratory virus (PRRSV) immune and naïve age-matched pigs were activated and treated with IL-21 and then tested for memory cell differentiation using a PRRSV non-structural protein 7 ELISPOT and ELISA. PRRSV immune pigs were positive on both ELISPOT and ELISA while naïve animals were negative on both assays. These results highlight the IL-21-driven expansion and differentiation of memory B cells *in vitro* without stimulation of the surface immunoglobulin receptor complex, as well as the establishment of a defined memory B cell culture system for characterization of vaccine responses in outbred animals.

2.2 Introduction

The memory B cell is a critical component of protective long-term immunity against reinfection. Following antigenic recognition, its ability to rapidly proliferate and differentiate into antibody secreting cells (ASC) results in the production of antigen-specific antibodies. These antibodies are essential for binding and clearance of invading pathogens prior to the incidence of clinical disease. Previous *in vitro* work in the pig has shown that this secondary humoral immune response requires antigen specific T cell help (Bergamin et al., 2007; Bryant et al., 2007). However, the factors necessary to stimulate robust porcine B cell expansion and differentiation to ASCs have not been extensively studied, except in a mixed leukocyte culture system (Crawley et al., 2003; Mulupuri et al., 2008). Work on human and mouse B cells has shown that, while many cytokines are capable of producing a proliferative and differentiating response, IL-21 is the most potent at driving this response (Li et al., 2015).

Interleukin-21 (IL-21) plays a key role in B cell biology, including the ability to robustly proliferate and differentiate activated naïve, germinal center, and memory B cells (Bryant et al., 2007; Ettinger et al., 2005; Kuchen et al., 2007; Lindqvist et al., 2012). It also has implications in pathological sequelae in the development of autoimmunity, rheumatoid arthritis, and transplant rejection (McGuire et al., 2011; Petrelli et al., 2011; Young et al., 2007). Collectively, this work has resulted in an enhanced understanding of how the adaptive immune system responds to antigenic recognition while also shedding light on the pro-inflammatory effects of IL-21. However, all previous research on IL-21 function has been limited to the mouse and human, resulting in a gap in knowledge of the function

of IL-21 in outbred animal models including animals which are important for nutrition, food and fiber.

The pig is a critical model species for biomedical research in diabetes and islet transplantation while at the same time is susceptible to a multitude of pathogens for which the memory immune response has not been characterized (Renner et al., 2016). The use of the pig for research and the ability to develop vaccines which stimulate an effective memory response have previously been hindered by a limited understanding of the factors which drive B cell differentiation. To date, the role of IL-21 in the pig adaptive immune response has not been investigated. Failure to understand the function of IL-21 on the pig B cell has prevented development of *ex vivo* strategies for evaluating protective memory responses to devastating pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV) a rapidly mutating RNA virus. Furthermore, a deficient understanding of the roles of important cytokines in porcine B cell biology has obstructed advances in the translational study of diabetes and transplantation immunology.

Here, we investigated the effects of IL-21, along with several other cytokines and factors (CD40L, IL-4, BAFF, APRIL) on CD21-positive porcine B cells. CD21 was used as a B cell marker due to its expression on all mature B cells, including memory B cells (Sinkora et al., 2013). These studies utilized an *in vitro* system to evaluate the effect of cytokines on mature B cell activation, proliferation, viability, and differentiation to ASCs. Finally, IL-21 was evaluated for its ability to proliferate and then differentiate PRRSV non-structural protein 7 (nsp7) specific memory B cells into antigen-specific ASCs. Our results demonstrate the proliferative and differentiating effects of IL-21 in

porcine B cells, reveal the roles of BAFF and APRIL for inhibiting porcine ASC apoptosis and maintaining cellular viability, and confirm a previous finding of a species-dependent difference of the B cell stimulatory effect of IL-4. It is now possible to establish optimal culture conditions for the expansion, differentiation, and evaluation of porcine memory B cells to specific antigens that can inform the role memory B cells in controlling specific diseases and serve as an outbred model for human immune responses to infectious diseases.

2.3 Materials and Methods

Antibodies and Reagents

The following antibodies were used: unlabeled goat anti-pig IgM and IgG polyclonal antibodies (Ab) (Bethyl), mouse anti-porcine IgM monoclonal antibody (mAb) (AbD Serotec K52 1C3), hamster anti-mouse CD80 PE-Cy7 mAb, mouse anti-pig SLA Class II DR mAb (AbD Serotec 2E9/13), horseradish peroxidase (HRP)-conjugated goat anti-pig IgM and IgG polyclonal Ab (Bethyl); phycoerythrin (PE)-labeled mouse anti-porcine CD21 mAb (Acris Antibodies BB6-11C9.6); FITC-goat anti-pig IgG polyclonal Ab (Bethyl); and eFluor 780-Fixable Viability Dye (eBioscience). Unlabeled antibodies were conjugated with the following APEX antibody labeling kits: Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647, and Pacific Blue. Reagents used for tissue culture included recombinant human (rh) BAFF and APRIL (Peprotech); IL-4 (Gibco); CD40L (BioLegend), and IL-21 (eBioscience). Cells were cultured in complete RPMI 1640 with

L-glutamine, 10% fetal bovine serum (FBS), 10 mM HEPES pH 7.2, 1% non-essential amino acids, 1% sodium pyruvate, and 20 µg/ml gentamycin.

Isolation and culture of CD21+ porcine B cells

Spleens from naïve or PRRSV-infected pigs were procured via tissue sharing with other investigators at the University of Minnesota. Dissociation of splenocytes from structural tissue was achieved by forceful pressing of splenic tissue pieces through a metal screen into culture media. Resulting cells and media were collected and passed over a 40 um filter. Cells were pelleted gently, and treated with ACK lysis buffer (Lonza) to remove red blood cells. Remaining cells were washed with ice cold PBS and retreated with lysis buffer. Cells were washed again, counted, put into freezing media (50% FBS, 40% complete RPMI 1640 media, and 10% DMSO) at ~10 million cells/ml, and frozen in liquid nitrogen following several cooling steps.

For experiments, cells were thawed from liquid nitrogen and magnetically enriched as described by Crawley et al. (Crawley et al., 2003). Briefly, cells were labeled with mouse anti-porcine CD21-PE. Miltenyi-Biotec mouse anti-PE microbeads were added and the suspensions were passed over consecutive Miltenyi-Biotec LS columns. CD21+ enriched cells were suspended in culture media, counted, assessed for viability and enrichment (>98%), and then placed into 96-well round bottom non-adhesive culture plates (Sarstedt) for 7 days at 50,000 cells/well. Cytokines, when used, were included at the following concentrations: CD40L (1.0 ug/ml), IL-4 (50 ng/ml), IL-21 (50 ng/ml), BAFF (100 ng/ml), APRIL (100 ng/ml). Concentrations were based off of previous literature and optimized for biological activity in our *in vitro* system (de Masson et al., 2015; Ding et

al., 2013; Good et al., 2006; Granato et al., 2014; Murtaugh et al., 2009; Saini et al., 2014).

Flow Cytometry

LSR Fortessa (BD Biosciences) and LSRII (BD Biosciences) instruments were used for flow cytometry. FACSDiva Software (BD Biosciences) was used for data acquisition. Data analysis was performed with FlowJo Software V10 (Tree Star).

B cell proliferation analysis

Cellular divisions were assessed by tracking the dilution of intracellular CFSE. Purified $CD21^+$ B cells were labeled with CFSE and cultured in triplicate either alone, with CD40L, or with CD40L and test cytokines. Cells were analyzed for proliferation and viability at 7 days of culture by flow cytometry. Total cell numbers for each condition were assessed in triplicate each day using trypan blue staining and a hemocytometer.

Intracellular Ig and Annexin V staining

Cell cultures were treated with 1X Brefeldin A Solution for one hour, then taken out of culture and resuspended in MACS buffer. Fixable viability dye (FVD) and Pacific blue labeled anti-IgM and IgG antibodies were added to the staining cocktail in order to coat and detect surface immunoglobulin. These cells were then fixed and permeabilized with a Cytofix/Cytoperm kit (BD). Following fixation and permeation, cells were washed with Cytofix/Cytoperm wash buffer. The same anti-IgM and IgG antibodies that were used in

the previous step, but conjugated to APC, were then used to stain intracellular immunoglobulin. Finally, cells were washed twice with Cytofix/Cytoperm wash buffer and evaluated via flow cytometry.

For Annexin V staining, cells were stained with the same extracellular antibodies and FVD as previously described for extracellular staining. Following EC staining, cells were washed with PBS, resuspended in 1X Annexin V binding buffer (FITC Annexin V apoptosis detection kit I, BD), and stained with Annexin V according to the manufacturer's protocol. Cells were washed with 1X binding buffer and resuspended in Cytofix/Cytoperm supplemented with 12.5 mM $CaCL_2$ (Trotter et al., 1995). 10X Cytofix/Cytoperm wash buffer was then diluted to 1X in Annexin V binding buffer and used for all subsequent washes and remaining IC staining.

Quantification of IgM and IgG secretion

Total secreted IgM and IgG in culture supernatants were assessed using a standard sandwich ELISA with goat anti-porcine IgM and IgG unlabeled polyclonal Abs to bind isotype specific immunoglobulins. Horseradish peroxidase conjugated goat anti-pig IgM and IgG antibodies were used to detect the presence of bound Ab. ELISA plates were read with a Bio-Tek plate reader at OD450 and analyzed with Gen5 2.05 software (Bio-Tek).

ELISPOT assay

ELISPOT was performed as described with the following modifications and using porcine reagents (Jahnmatz et al., 2013; Mulupuri et al., 2008). Plates were coated with 0.5 ug/well of nsp7 or anti-pig IgG polyclonal antibody, and HRP-conjugated anti-pig IgG polyclonal antibody was used as the detection reagent. Also, following AEC addition and washing, the back of the ELISPOT plate was removed and the backsides of the membranes were washed with tap water to remove unbound dye and eliminate background staining of the membrane.

2.4 Results

CD40L as an activator of porcine B cells

To investigate the ability of CD40L to activate porcine B cells we magnetically enriched $CD21^+$ splenocytes and cultured them for one week in the presence or absence of soluble human CD40L (CD154). Human CD40L binds porcine CD40 on pig endothelial cells and induces biological activity (Rushworth et al., 2000, 2001). Expression of CD80 and MHCII was evaluated using flow cytometry every 24 hours on both CD40L treated and untreated cells. Both CD80 and MHCII have previously been used as activation markers for mouse, human and porcine B cells (Ahmadi et al., 2008; Chaperot et al., 1999; Mizrahi et al., 2013; Takamatsu et al., 1999; von Bergwelt-Baildon et al., 2002). CD40L treatment resulted in a seven-fold increase in MFI of MHCII expression by day 2 of culture. This significant difference in MHCII expression between treated and untreated cells gradually declined to levels observed on live unstimulated cells by day 6 (Fig 1A).

CD80 expression was drastically reduced on all B cells when put into culture. However, live cells that were treated with CD40L maintained higher levels of CD80 expression for the first 6 days of culture (Fig 1B).

B cell activation is necessary for robust IL-21 stimulation

Previous work with porcine B cells showed that cellular activation with CD40L induced low levels of proliferation in certain subsets of B cells and that activation was necessary for many stimulatory cytokines to elicit an effect (Takamatsu et al., 1999). During initial cultures, comparing the activation of $CD21^{+}$ cells, we also noticed a difference in cellular proliferation between CD40L-activated and non-activated cultures. Therefore, we monitored proliferation over 7 days of culture after labeling cells with CFSE. Additionally, we tested the effect of two purported B-cell stimulatory cytokines, IL-4 and IL-21, on porcine B cells to determine whether activation was necessary for stimulatory cytokines to elicit a proliferative and differentiating response.

In the absence of CD40L, IL21 induced a low level (~10%) of proliferation, whereas IL-4 showed no activity (Figs 2A and 2B). Following CD40L activation, IL-21 induced a potent proliferative response and an approximately 2.5-fold increase in total cell number (Figs 2B and 2C). Most cellular divisions occurred between 24 and 72 hours of culture evidenced by the sharp rise in live cells during this time period (Fig 2D). These cells appeared to be short lived, as there were few viable cells left in culture by day 7. IL-4 displayed no proliferative effect in the presence of CD40L activation (Fig 2B).

IL-21 has been reported in the human and mouse to be important for B cell differentiation into antibody secreting cells (Avery et al., 2010; Bryant et al., 2007; Ozaki et al., 2002). After 7 days of porcine B cell culture in the absence of CD40L, no IgM was detected (Fig. 2E). CD40L alone resulted in approximately 200 ng IgM per well. By contrast, inclusion of IL-21 to CD40L-activated B cells resulted in maximal IgM production in the range of 3-4 µg per well (Fig 2E).

IL-4 was discovered as B cell stimulatory factor in the mouse and human (Hasbold et al., 1999; Lee et al., 1986). However, past work with both porcine and human IL-4 in a porcine splenocyte culture system showed that it elicited no stimulatory effect outside of acting as a potential maintenance factor for activated B cells (Murtaugh et al., 2009). In purified B cell cultures activated with CD40L we also found that IL-4 had no impact on proliferation (Fig 2B), on IgM production (Fig 2E), or on viability in the presence or absence of CD40L (Fig 2F).

BAFF as a maintenance factor for porcine B cells

The decline of culture viability following IL-21 driven expansion, shown in Fig 2D, suggested that maintenance factors, such as BAFF and APRIL, might enhance cellular survival and function. BAFF and APRIL, members of the TNF family of ligands, are important for driving porcine B cell proliferation for certain subsets of B cells and ASCs (Guan et al., 2007; Shui et al., 2008). Treatment with BAFF and APRIL on non-activated, CD40L treated, and IL-21 stimulated cells did not result in an increase in cell numbers over seven days of culture, demonstrating that they did not affect B cell proliferation *in vitro* (Fig 3A). However, BAFF significantly enhanced cellular viability for both non-

activated and CD40L activated B cells when compared to untreated cells and to cells treated with APRIL (Fig 3B). Treatment of cultures with both APRIL and BAFF did not result in significant differences in viability from treatment with BAFF alone. However, BAFF and APRIL together enhanced viability in cultures stimulated with CD40L and IL-21 (Fig 3B).

As noted previously, IL-21 induced marked proliferation of B cells activated by CD40L binding. However, proliferating cells died rapidly, with a half-life of about 2 days (Fig 2D). BAFF and APRIL significantly increased proliferating cell viability, as shown in Fig. 3B, with functional consequences of increased antibody secretion of both IgM and IgG (Figs 3C and D).

BAFF and APRIL as maintenance factors for ASCs

Although BAFF and APRIL did not affect B cell proliferation or differentiation, they have been shown to promote survival of ASCs in several species (Avery et al., 2003; Benson et al., 2008; Bergamin et al., 2007). Therefore, we cultured CD40L activated $CD21^{+}$ B cells with IL-21 and BAFF and APRIL and then evaluated cells every 24 hours for 7 days via flow cytometry for intracellular IgM and IgG in live cells. As shown in Figure 4A, the addition of IL-21 alone to CD40L activated cells resulted in the differentiation of approximately 8% of all B cells to IgM ASCs. There are two populations of live IC IgM+ cells displayed in figure 4A. The IC IgM bright population is identified as IgM ASCs based on antibody production per well across treatments. The IC IgM dim population is likely a germinal center, non-antibody secreting, phenotype similar to that which has been observed in mice (Pape et al., 2011). Time course analysis

showed that IgM-positive cells were maximally induced within the first 24 hours of culture and BAFF and APRIL enabled a more sustained maintenance of positive cells (Fig 4B). IgG ASCs peaked at 48 hours of culture in CD40L activated and IL-21 differentiated cells, but the addition of APRIL and, especially, BAFF, maintained IgG-positive ASCs for up to 96 hours (Fig 4C), leading to a nearly two-fold increase in both IgM and IgG production compared to cultures without the maintenance factors (Figs 4D and 4E). BAFF appeared to be more effective in enhancing IgM production, as compared to APRIL, but there was no difference in IgG secretion (Figs 4D and 4E). No difference in total cell numbers was noted for any cultures treated with IL-21, ruling out APRIL or BAFF driven differences in antibody secretion due to effects on cellular proliferation (Fig 4F).

Lastly, culture viability was assessed using a fixable viability dye with flow cytometry and total cell counts at day 7 of culture. As shown previously in Fig. 2D, IL-21 inclusion reduced total cell viability after 7 days compared to CD40L alone. However, APRIL restored IL-21-treated cultures to the level of CD40L alone and BAFF significantly enhanced live cell numbers (Fig 4G). Treatment with APRIL and BAFF together did not result in a statistically significant difference in viability over treatment with BAFF alone (Fig 4G). Overall, we have found that inclusion of both APRIL and BAFF on CD40L activated and IL-21 stimulated cells consistently results in numerical enhancement, which is not statistically significant, of cellular viability and antibody secretion as compared to treatment with either one alone.

Maintenance factors inhibit ASC apoptosis

The increase in ASC viability and antibody production observed with maintenance factor treatments led us to hypothesize that BAFF and APRIL were affecting cellular viability through the inhibition of ASC apoptosis. Hence, we labeled cells with a fixable viability dye, followed by annexin V staining, and then fixed and permeabilized the cells to detect intracellular (IC) IgM. IC IgM^+ cells separated into two distinct populations displaying both bright and dim fluorescence associated with annexin V binding (Fig 5A). Gating on these populations showed that the majority of annexin V bright ASCs did not have intact plasma membranes (fixable viability dye, FVD^+) and were thus considered non-viable (Fig 5B). The majority of annexin V dim cells maintained an intact plasma membrane (FVD^-) and were still viable (Fig 5C). Treatment with IL-21, in the absence of a maintenance factor, resulted in elevated numbers of non-viable annexin V-bright ASCs and decreased numbers of viable annexin V dim cells as compared to IL-21 treated cultures which also received BAFF or APRIL, or both, as shown in Fig 5A. These results show that the positive effects of BAFF and APRIL on ASC viability and enhanced antibody production observed in Figs 4D and 4E are due at least in part to their ability to inhibit ASC apoptosis.

IL-21 stimulates proliferation and differentiation of memory B cells

The identification of IC IgG+ cells at 48 hours of culture led us to hypothesize that IL-21 was inducing the proliferation and differentiation of activated memory B cells into ASCs. Thus, we evaluated the memory response of naïve and immune animals to PRRSV nsp7. The function of nsp7 is unknown, but it is highly immunogenic, thus increasing our

opportunity to detect rare antigen specific memory B cells (Brown et al., 2009; Mulupuri et al., 2008). Splenocytes were isolated from two non-exposed, negative and four immune mature female pigs that were infected with virulent field viruses >28 days prior to sacrifice. Cells were enriched for CD21, labeled with CFSE, and then treated with CD40L and the maintenance factors APRIL and BAFF to enhance ASC viability. IL-21 was added to half of the tissue culture wells and on day 3, at the peak of the *in vitro* IgG antibody response, equal aliquots of cells were used for proliferation analysis, transferred to PRRSV nsp7 and IgG ELISPOT plates, or were left in culture until day 14, when supernatants were harvested to test for nsp7-specific IgG by ELISA.

To track proliferation, cells were analyzed by flow cytometry for cell division and IC IgG staining. B cells treated with IL-21 differentiated into IC IgG^+ ASCs, but only after having undergone several rounds of division (Fig 6A). ELISPOT analysis showed equivalent numbers of IgG-secreting ASC in all animals after, but not before, stimulation with IL-21, and nsp7-specific antibody secreting cells only in immune animals (Fig 6B). Since the number of spots in the ELISPOT assay potentially exaggerated the proportion of IgG^+ nsp7-specific memory B cells initially isolated from the spleen, all cultures were assayed both for nsp7-specific ASC differentiated from memory B cells and for IgG ASC to control for proliferation. All four PRRSV-immune pigs had memory B cells against nsp7, with more than a 20-fold difference between minimum and maximum frequency (Fig 6C). PRRSV naïve pigs were completely negative on nsp7 ELISPOTs. Antigen-specific ELISPOT results were supported by nsp7 ELISAs which showed three immune pigs with endpoint dilution titers of 1:8. Naïve control pigs and immune animal #4, with minimal ELISPOT values, showed background absorbance levels on ELISA (Fig 6C).

2.4 Discussion

IL-21 plays a critical role in the adaptive immune response due to its ability to stimulate B cell differentiation and antibody production, and to promote the maladaptive development of inflammatory disease and autoimmune disorders, such as type 1 diabetes (Sutherland et al., 2009; Walker and von Herrath, 2016). However, limited data on IL-21 activities in the pig hinders progress in development of the pig for organ and islet xenotransplantation. It is of particular importance for type 1 diabetes, as IL-21 production is increased in type 1 diabetic patients, and resulting B cell involvement in disease progression seems likely (Ferreira et al., 2015; Leeth et al., 2016). Furthermore, the cross-species activities of human CD40L and IL-21 on porcine B cells highlights the unanticipated host-graft interactions that may complicate xenotransplant acceptance.

Purified B cells placed into standard cell culture media die rapidly, indicating that their survival in the animal is dependent on cellular and molecular interactions in lymphoid tissues. CD40L activation by itself induces MHC II expression, makes cells permissive to IL-21, and increases cell half-life, but did not change B cell fate. IL-21 by itself had no apparent effect on survival, but in the presence of CD40L, it stimulated a robust proliferative differentiation of short-lived cells. The rapid decline in viability was consistent with an apoptosis inducing activity of IL-21, which has been previously described (Jin et al., 2004; Mehta et al., 2003). In our hands, BAFF, but not APRIL, promoted resting B cell survival, suggesting that it was mediated through the BAFF-R as opposed to their shared receptors, transmembrane activator and CAML interactor (TACI)

and B cell maturation antigen (BCMA). Both BAFF and APRIL were able to increase ASC viability and enhance production of both IgM and IgG by inhibiting apoptosis. These findings are consistent with other studies showing that BAFF/APRIL support plasmablasts derived from rapidly dividing memory B cells (Avery et al., 2003; Belnoue et al., 2008; Bergamin et al., 2007). The specific receptor interactions mediating these effects may be dependent upon the type and location of the ASC (Bossen et al., 2008; O'Connor et al., 2004; Peperzak et al., 2013; Tsuji et al., 2011).

Kinetic analysis showed that numbers of live IgM^+ ASCs were highest within 24 h of B cell activation and stimulation and invariably declined after 48 h. IgG^+ ASCs are a minor population that peaks at 48-96 h after stimulation, suggesting that they represent the memory B cell phenotype. This is quicker than has been observed in cultures of human class switched B cells from the spleen which peaked between days 3 and 4 of culture (Bryant et al., 2007). Comparison of nsp7-specific memory B cell ELISPOT and secreted antibody titers from the culture wells shows agreement supporting the conclusion that activated and proliferating memory B cells are the source of antibody production.

The lack of a stimulatory effect of IL-4 on porcine B cells is consistent with previous findings that IL-4 does not induce proliferation of porcine B cells (Murtaugh et al., 2009), in stark contrast to its role in the mouse (Howard et al., 1982). Additionally, its inability to affect B cell viability, as had been previously proposed, suggests that IL-4 may be unimportant in porcine B cell biology. We did not investigate if IL-4 may play a role in class switching to IgE, IgA, and subclasses of IgG (Avery et al., 2008; Ramadani et al., 2016; Seidl et al., 2012).

The toolkit for defining B cell differentiation stages in the pig is smaller than in mice and humans. Antibodies for CD19 have not been characterized, and CD27 is not expressed on porcine B cells (Reutner et al., 2012). Therefore, we defined memory cells by CD21 positivity and antigen-specific functional analysis for PRRSV nsp7. The displayed ability of the T cell associated protein, CD40L, and IL-21, to proliferate and differentiate memory cells supports previous work showing that T cell help is necessary for in vitro activation and differentiation of memory B cells (Bergamin et al., 2007). By using IL-21, along with other T cell and dendritic cell cytokines and factors, we were able to expand and differentiate memory B cells in the absence of potentially confounding secondary cell types and cytokines. Furthermore, the exhibited ability of IL-21 to elicit a robust memory B cell proliferation and differentiation response in the absence of antigen and BCR engagement is congruent with previous work which utilized mixed splenocyte-cultures (Mulupuri et al., 2008). These findings are consistent with previous results in human lymphocytes which first described the “bystander” effect and may be important for porcine maintenance of memory populations or autoimmune disease development (Bernasconi et al., 2002; Lanzavecchia, 1983).

In conclusion, we demonstrate that IL-21 is a potent stimulator of CD40L activated porcine B cells, and that it is able to proliferate and differentiate rare memory B cells. It is now possible to address the molecular and cellular bases of immune response variation that confounds human and porcine vaccination efficacy using a relevant outbred species model.

2.5 Acknowledgements

We thank Diem Ngo for technical assistance. The research was supported by USDA NIFA grant 2015-06966 and an NIH fellowship to MCR from the training grant T32 OD010993.

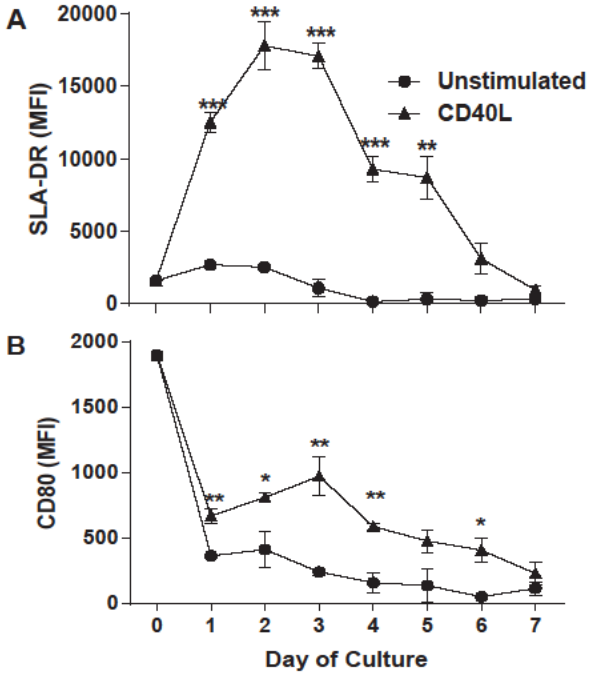

Figure 2-1 CD40L induces B cell activation

Figure 2.1 - CD40L induces B cell activation. $CD21^{+}$ purified B cell populations were cultured in the presence or absence of CD40L (1.0 ug/ml), gated on live cells, and then analyzed for changes in the median fluorescence intensity (MFI) of SLA-DR (A) and CD80 (B). Cells were analyzed prior to culture and at 24 h intervals for a period of 7 days. One representative experiment of two performed is displayed. Data shown are mean ± SEM. Statistical differences of *$p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$ are indicated.

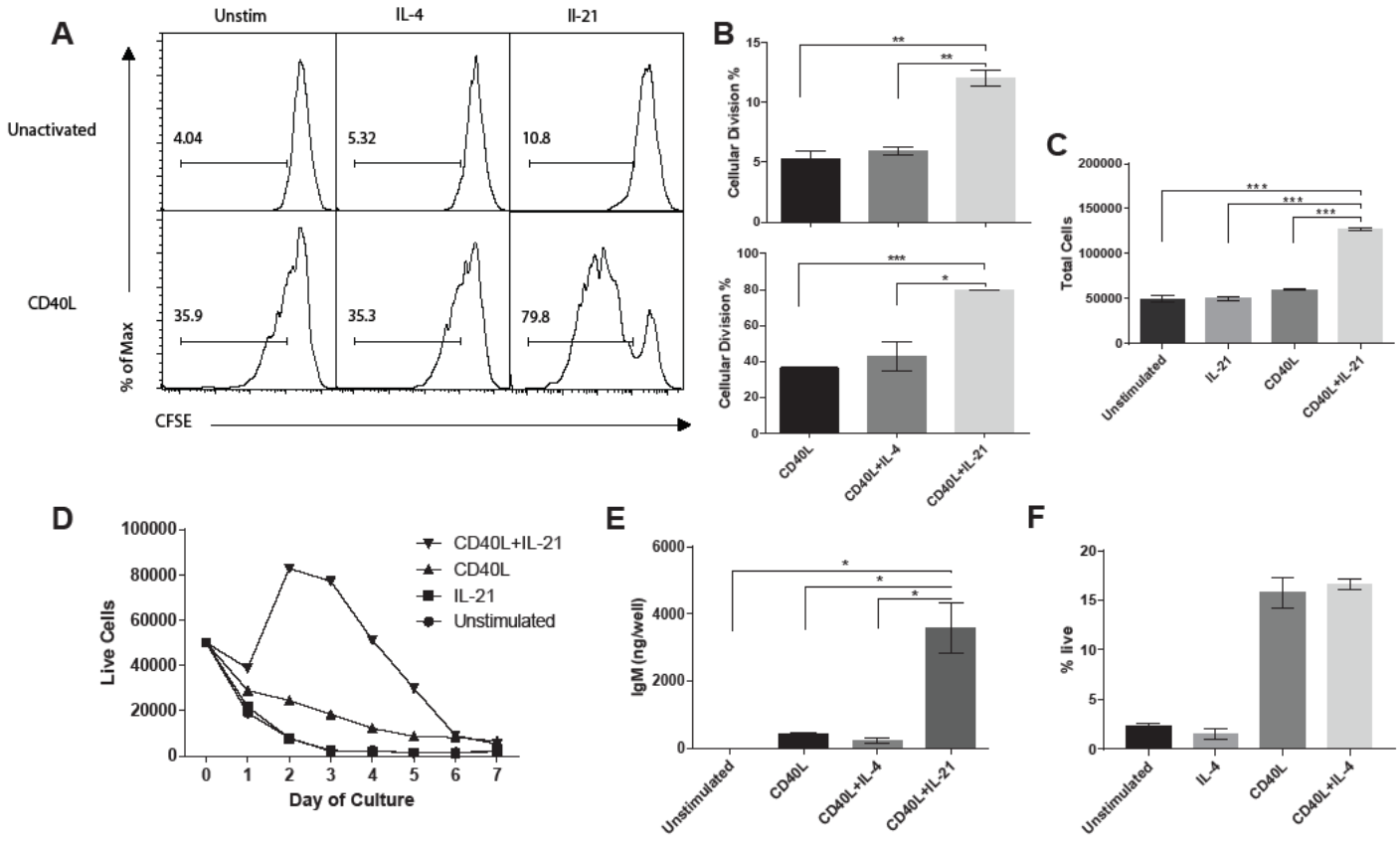

Figure 2-2 IL-21 is a potent inducer of CD40L-activated B cell proliferation and differentiation

Figure 2.2 - IL-21 is a potent inducer of CD40L-activated B cell proliferation and differentiation. Quiescent or CD40L activated $CD21^+$ cells were stimulated with IL-4 or IL-21 at 50 ng/ml. (A) $CD21^+$ cells were stained with CFSE and analyzed by flow cytometry at day 7 of culture for proliferation. (B) Proliferating percentages for each treatment condition. (C) Total cell numbers after 7 days of culture. (D) Cell culture viability dynamics. (E) IgM production from activated and stimulated cells, quantified with a total IgM ELISA of culture supernatant. (F) Cell viability evaluated by fixable viability dye staining and flow cytometry at day 7. One representative experiment of three with three different animals is displayed. Data are mean ± SEM. Statistical differences of $*p < 0.05$, $** p < 0.01$, $***p < 0.001$ were determined using ANOVA to compare group means followed by t-tests to calculate significance.

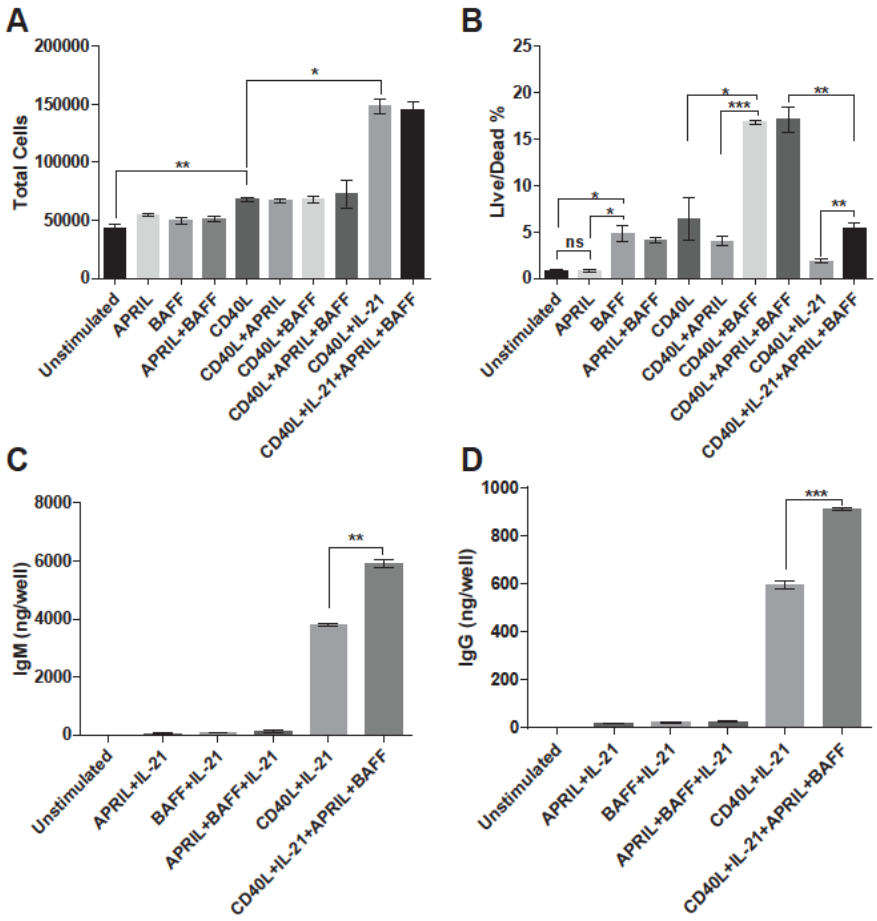

Figure 2-3 BAFF is a maintenance factor for porcine B cells

Figure 2.3 - BAFF is a maintenance factor for porcine B cells. CD21+ purified B cells were treated with cytokines as described and cultured in technical triplicates. Culture supernatants and cells were collected and analyzed at day 7 for number of total cells via hemocytometer (A) and flow cytometry with fixable viability dye (B). Total secreted IgM (C) and IgG (D) were determined by ELISA as described in Materials and Methods. Results are representative of three independent experiments with three individual animals. Data are mean ± SEM. Statistical differences of *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ were determined using ANOVA to compare group means followed by t-tests to calculate significance.

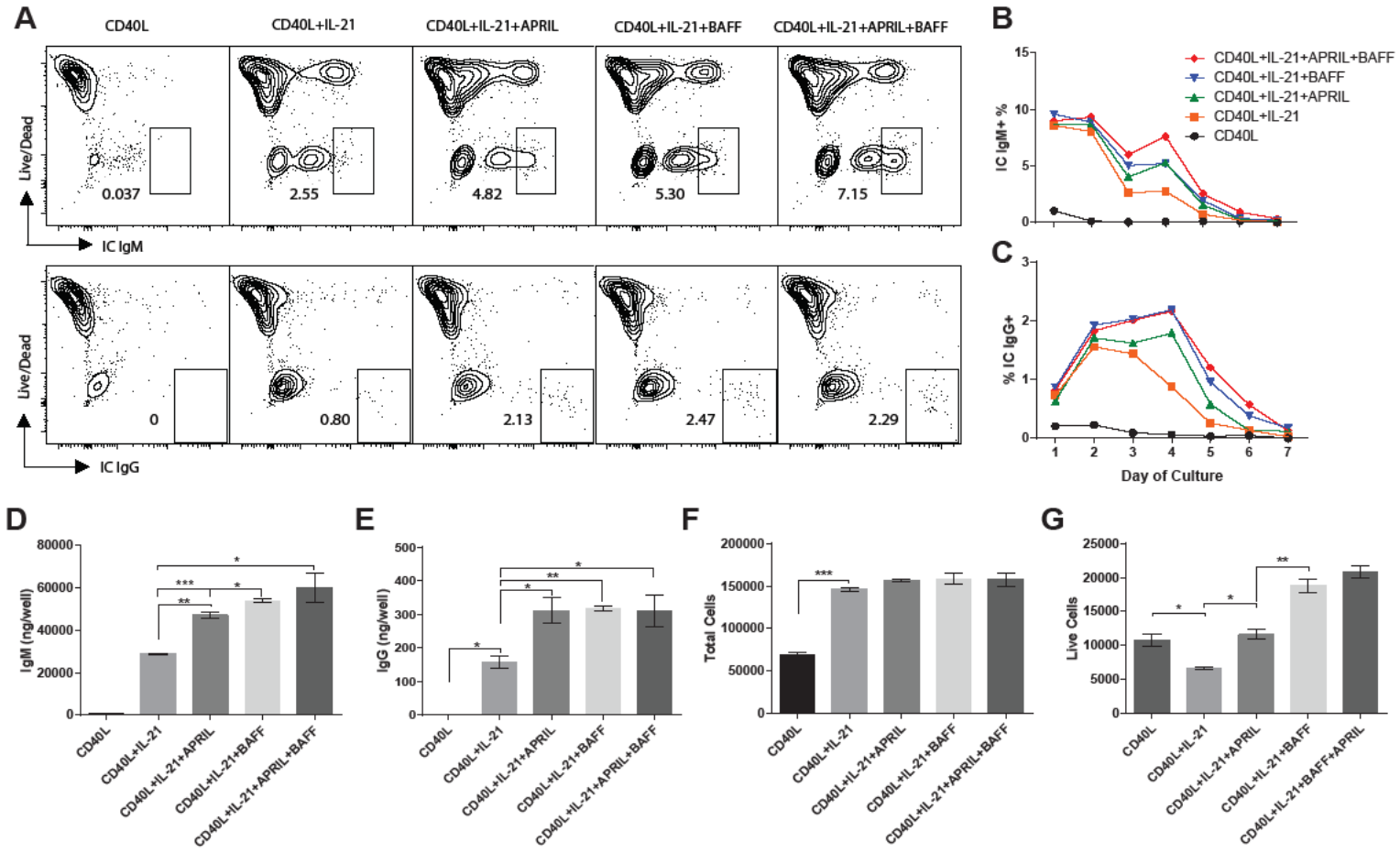

Figure 2-4 BAFF and APRIL support ASC viability

Figure 2.4 - BAFF and APRIL support ASC viability. $CD21^+$ B cells were cultured in triplicate in the indicated cytokines to determine the effects on IgM and IgG ASC differentiation at 4 days of culture (A). Cells were evaluated at 24 h intervals up to 7 days for live IC IgM^+ (B) and IgG^+ (C) cell percentages via flow cytometry, using the gating strategy and results for day 4 of culture displayed in panel A. On day 7, culture supernatants and cells were collected and analyzed for secreted IgM (D) and IgG (E) by ELISA. Total cells (F) were counted using a hemocytometer. Fixable viability dye and flow cytometry were used to determine cellular viability and then multiplied by total cell counts to calculate total live cell counts (G). Data (mean ± SEM) shown are from one of three equivalent experiments with three different animals. Statistical differences of $*p < 0.05$, $** p < 0.01$, $***p < 0.001$ were determined using ANOVA to compare group means followed by t-tests to calculate significance.

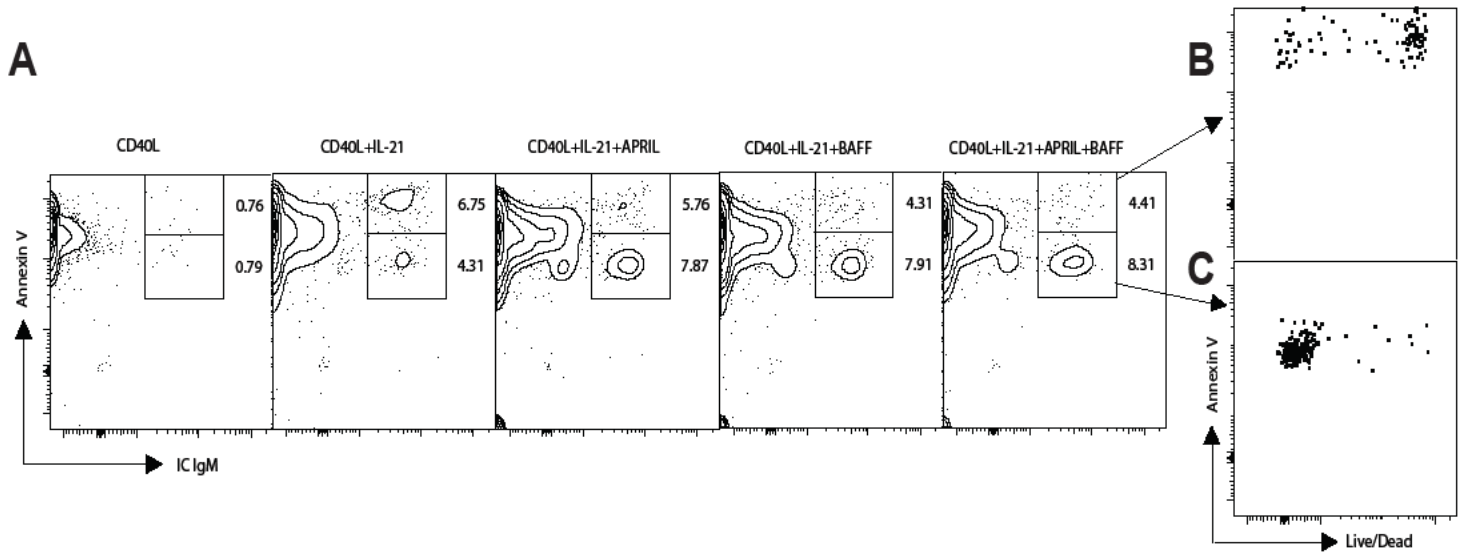

Figure 2-5 BAFF and APRIL extend ASC viability by delaying apoptosis

Figure 2.5 - BAFF and APRIL extend ASC viability by delaying apoptosis. $CD21^{+}$ cells were cultured in triplicate in the indicated cytokines. On day 4 of culture, cells were incubated with unlabeled anti-IgM, then stained for flow cytometry with a fixable viability dye, CD21, Annexin V, and then fixed and permeabilized and stained for IC IgM (A). Annexin V bright (B) and dim (C) populations were gated to show the live and dead distribution of the two populations. One representative experiment of two performed with two different animals is displayed.

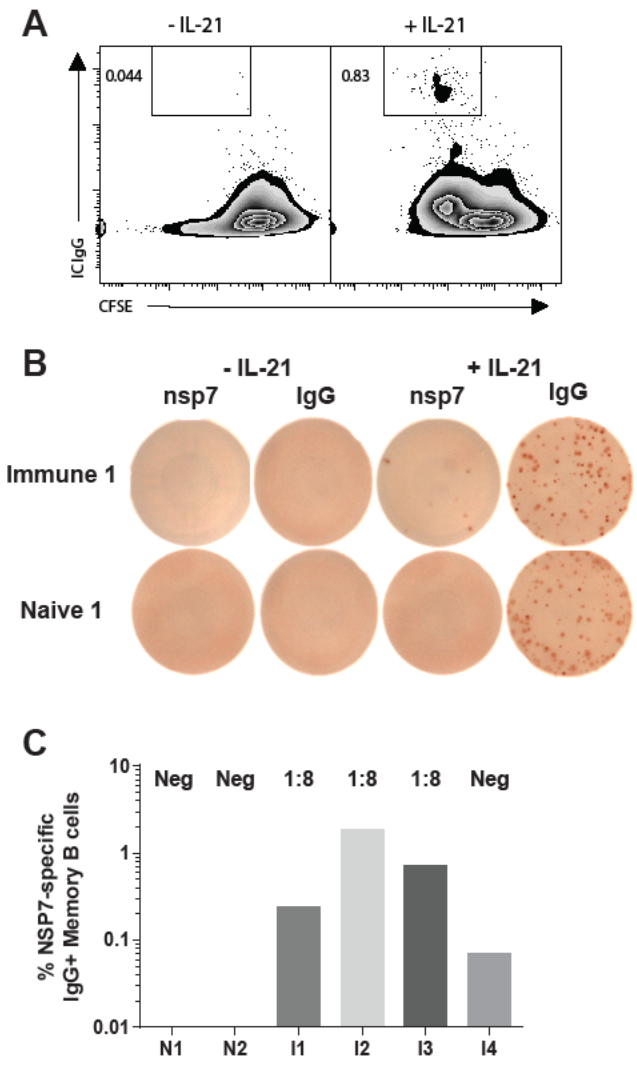

Figure 2-6 IL-21 proliferates and differentiates memory B cells

Figure 2.6 - IL-21 proliferates and differentiates memory B cells. Splenocytes from four PRRSV immune and two naïve animals were enriched for $CD21^+$ B cells. (A) Cells were labeled with CFSE and then cultured in the presence of CD40L, APRIL, and BAFF with or without IL-21. At day 3 of culture, 1/3 of cells were harvested and evaluated for IC IgG^+ and CFSE divisions via flow cytometry. (B) PRRSV nsp7 (100,000 live cells/well) and IgG^+ ELISPOT (10,000 live cells/well) were performed on 1/3 of cultured cells. Following normalization of plated cell number, the proportion of nsp7-specific spots to IgG^+ spots was calculated and is displayed in (C). The remaining IL-21 treated cells were cultured for a total of 14 days. Supernatants were harvested and evaluated with a limiting dilution nsp7 ELISA. Individual titers are displayed above corresponding columns in (C). Data are representative of two independent experiments with the same animals.

Chapter 3 – PRRSV nsp7 B cell tetramer development and validation

A manuscript submitted to Viral Immunology

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3.1 Abstract

Immunological memory is elicited following either vaccination or natural exposure to a pathogen and is essential for protection against re-exposure. Despite its critical importance, the ability to interrogate the veterinary animal memory immune response has long been hindered by a paucity of tools to assess immunological memory. As a result, the evaluation and analysis of protective immune responses that predict immune protection in food and fiber animals and facilitate vaccine development are obstructed. To fill this gap in knowledge in swine, we created a B cell tetramer to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural protein 7 to efficiently and effectively investigate the memory B cell response, a hallmark of anti-viral immunity. This novel reagent was validated using a modified capture ELISA, tetramer pulldowns and flow cytometry, and was shown to detect rare, antigen-specific B cells present at a frequency of about 0.001% of total B lymphocytes in immune animals. The nsp7-B cell tetramer will help to characterize the PRRSV-specific memory B cell response which is fundamentally important for understanding immunological competence and animal variation in resistance to PRRSV infection. We expect that the method will be widely applicable to exploration of immunity to veterinary pathogens.

3.2 Introduction

The memory immune response is an essential component of adaptive immunity to infectious diseases. Following initial antigen recognition and response, antigen-educated lymphocytes differentiate into long-lived memory cells that respond rapidly and potently to a later exposure to the same pathogen. This recall response is also the mechanism behind booster vaccinations: low affinity memory B cell recognition of antigen induces proliferation, differentiation and hypersomatic mutation into B cells whose high affinity antibodies are more efficient at antigen recognition and pathogen control. Since antibodies are key effectors of immune protection for the majority of anti-viral vaccines, memory B cells are essential for the development of effective vaccinal immune protection (Burton, 2002; Plotkin, 2010). Hence, tools for the assessment of memory B cell status and characteristics are needed to facilitate a better understanding of veterinary infectious diseases and mechanisms of vaccine efficacy.

Previous attempts to analyze the memory B cell response to vaccination or viral exposure in veterinary species have been performed using enzyme linked immunospot assays (ELISPOTs) (Mulupuri et al., 2008; Rahe and Murtaugh, 2017b). While this is an effective test for identifying the presence of memory cells within tissues, it is limited by laborious cell isolation and culture procedures in a laboratory, and the inability to determine the frequency of memory cells in vivo due to the necessary proliferation and differentiation of memory B cells which is required to detect antibody secreting cells (ASCs) (Crotty et al., 2004). In addition, the stimulation and culture of splenocytes and lymphoid tissue leukocytes includes plasma cells, which may result in over-estimation of the memory cells abundance in the tissue of interest.

B cell tetramers were developed to overcome these limitations (Newman et al., 2003). Tetramers consist of four identical biotinylated proteins linked to a streptavidin core that is bound to a bright fluorescent protein, such as phycoerythrin (PE). Surface immunoglobulins of antigen-specific B cells bind to the proteins of the tetramer, thus enabling their detection by flow cytometry. The result is a highly sensitive reagent capable of capturing rare antigen-specific memory B cells using flow cytometry in whole blood or shortly following cellular dissociation from tissue (Taylor et al., 2012b).

Understanding the memory immune response to porcine reproductive and respiratory syndrome virus (PRRSV) is particularly important since it is the biggest threat to pig health and wellbeing worldwide, and has extensive genetic plasticity. PRRSV is a rapidly mutating RNA virus notorious for unreliable control by vaccination. Even so, primary PRRSV infection is consistently and completely cleared from infected animals after prolonged periods of time by mechanisms which are not well understood (Schaefer and Morrison, 2007; Torremorrell et al., 2002). To date, the means for producing a broadly protective immune response in the pig have not been determined. The ability to efficiently and effectively analyze the porcine B cell memory response to PRRSV infection and vaccination with B cell tetramers will advance knowledge of the immunological interaction of PRRSV with pigs, and provide a model for similar investigations in other host-pathogen interactions.

3.3 Materials and Methods

Tissue collection

Tissues were procured via tissue sharing under the University of Minnesota IACUC protocol 1702-34568A.

Nonstructural protein 7 (nsp7) biotinylation and tetramer production

Soluble recombinant nsp7 protein from PRRSV2 strain VR2332 containing an amino-terminal myc tag and a carboxyl 6x-histidine tag (referred to hereafter as nsp7) was generated in *E. coli* bacteria and purified by cobalt immobilized metal affinity chromatography as previously described (Brown et al., 2009; Dvorak et al., 2016; Ferrin et al., 2004; Johnson et al., 2007). The molar concentration of nsp7 was determined by spectrophotometry, using an extinction coefficient of 0.036565 μM^{-1} cm^{-1} at 280 nm, with a Take3 plate and a Biotek Epoch microplate instrument.

Tetramer production was modified from Taylor et al. (Taylor et al., 2012b). Protein biotinylation was achieved using an EZ-link Sulfo-NHS-LC-Biotinylation kit (Thermo Fisher Scientific) at a ratio of 1.2 biotin molecules to 1 nsp7 molecule (Fig. 1). Free biotin was removed using 3K Amicon Ultra-0.5 ml centrifugal filters (Millipore). Biotinylation efficiency was evaluated by a quenching western blot. 10 pmol aliquots of nsp7 were incubated with two fold dilutions (0, 5, 2.5, 1.25, 0.625, 0.31, 0.156 pmol) of streptavidin (SA)-PE (Prozyme) at room temperature for 30 m. The mixtures were then

run on a native protein gel for 15 m at 150V. The gel was transferred to a nitrocellulose membrane via western blot for 1 h at 100V. The membrane was washed with PBS and blocked for one hour at room temperature with Odyssey blocking buffer (Licor). The membrane was washed with PBST and then incubated with SA-AF680 (Prozyme) at a concentration of 1:10,000 in PBST for 30 minutes. The membrane was washed twice with PBST and then read on a Licor Odyssey imager at an absorbance of 700 nm. The resulting level of biotinylation was determined by the reemergence of a band of biotinylated protein which was not fully quenched by the SA-PE and was able to react with SA-AF680.

Biotin labeling efficiency was determined based on the molarity of SA-PE when the band of biotinylated antigen reappeared, and multiplication by 4 to account for the four biotin binding sites on SA (Fig. 2). This number was divided by the molarity of the biotinylated antigen from the quenching reaction (i.e., 2 uM). Multiplying the quotient by 100 resulted in the percentage of antigen which is effectively biotinylated. A value between 25-75% is considered optimal. A conservative estimate of 25% was made in the example used here. Additionally, the quotient was multiplied by the molarity of the biotinylated antigen, the concentration of which had been diluted to give 2 uM. The result is the molarity of biotinylated antigen in the sample and is important for tetramer formation.

To fully arm the tetramer, 4 molar equivalents of biotinylated nsp7, calculated from the quenching western blot, were mixed with one molar equivalent of SA-PE and incubated for 1 h at room temperature and then purified with a 100K Amicon Ultra spin filter (Fig. 3). The concentration of retained tetramer was determined by measuring the absorbance at 566 nm with a Take3 plate and a Biotek Epoch spectrophotometer and adjusted by the

extinction coefficient for PE. The prepared nsp7 tetramer was diluted to a concentration of 2 uM for use.

A negative control decoy tetramer consisting of streptavidin-PE labeled with Alexa Fluor® 647 (AF647) (Thermo Fisher Scientific) was also created in the same manner as previously described (Krishnamurty et al., 2016; Taylor et al., 2012b).

nsp7 ELISA

Recombinant nsp7 was diluted in carbonate buffer, coated onto 96 well ELISA plates (Sarstedt) at 100 ng/well, and incubated overnight at 4 C. Plates were washed 3 times with PBS + 0.05% Tween-20 (PBST) in a Biotek plate washer. Wells were blocked with 5% non-fat dry milk (NFDM) diluted in PBST, pH 9.6, for 2 h. The plates were washed and incubated with 100 ul of immune or naïve serum diluted 1:50 in 5% NFDM, pH 7.4, for 1 h. Wells were washed and incubated with 1:100,000 dilution of polyclonal goat anti-pig IgG-HRP (Bethyl) in 5% NFDM, pH 7.4, for 1 h. Plates were washed and then developed with TMB peroxidase (KPL) for 15 minutes. The reaction was stopped with 100 ul of 1 M phosphoric acid. Plates were read at 450 nm with a Biotek Epoch microplate instrument.

Tetramer capture ELISA

One hundred µl of serum from PRRSV immune and naïve pigs was incubated with 2 pmol of nsp7 tetramer for 1 h at room temperature. Thirty µl of anti-PE microbeads (Miltenyi Biotec) were added and incubated for an additional 30 m at room temperature.

Three hundred and sixty µl of PBS were added to the mixture to a final volume of 500 µl. Tetramer enrichment was then achieved by passing the mixture over an LS column in a magnetic cell separator (Miltenyi Biotec). The LS column was washed with 3 ml of PBS three times. The column was then removed from the cell separator and placed in a 5 ml FACS tube. Two and one half ml of PBS was added to the column and magnetic beads bound to tetramer were eluted from the column with a forceful plunging action. The mixture was then transferred to 2 ml Eppendorf tubes and centrifuged at 16,000xg for 15 minutes. The resulting brown pellet contained microbeads bound to nsp7 tetramer and bound antibodies, if present. The supernatant was discarded and the pellet was resuspended in 1 ml of PBS. The microbead-tetramer-antibody mixture was centrifuged again at 16,000xg for 15 minutes. The supernatant was aspirated and the pellet was thoroughly resuspended in 200 ul of 5% non-fat dry milk at pH 7.4. This mixture was then analyzed for total IgG in a total IgG sandwich ELISA as previously described (Rahe and Murtaugh, 2017b).

Tetramer pulldown

Magnetic enrichment for tetramer bound to antigen-specific cells from the tracheobronchial lymph node was carried out as previously described with modifications (Fig. 4) (Krishnamurty et al., 2016; Taylor et al., 2012b). Briefly, eighty million live cells were thawed from each of one PRRSV immune and one PRRSV naïve animal. Cells were resuspended in MACS buffer (1x PBS, 2.5% FBS, 2 mM EDTA, 30 nM DNase 1) and centrifuged at 1,400 RPM for 5 minutes. Cells were resuspended in 100 ul of 7.5 nM decoy tetramer stain and incubated on the bench top for 10 minutes under foil. Cells were

resuspended in 10 ml of MACS buffer and filtered through a 40 um filter (Corning). Five hundred microliters of resuspended cells were removed for live cell count with a hemocytometer as well as flow staining for CD21 expression and viability to identify the starting number of live B cells. The remaining cells were centrifuged and then stained with 100 ul of 7.5 nM nsp7-tetramer stain. Cells were incubated for 30 min at 4 C. Following incubation, cells were washed with 10 ml of MACS buffer and then resuspended in 200 ul (160 ul MACS, 40 ul microbeads) of microbead mix (Miltenyi). Samples were incubated for 30 minutes at 4 C. Cells were washed with 10 ml of MACS buffer, centrifuged, and resuspended in 500 ul of MACS buffer. This cellular suspension was dispensed over a 40 um filter into an LS column (Miltenyi) resting in a QuadroMACS separator. The column was washed 3 times with 3 ml of MACS buffer. After washing, the column was removed from the separator and placed in a 5 ml FACS tube. Three ml of MACS buffer were added to the column and forcibly plunged into the FACS tube to elute cells from the column. Eluted cells were centrifuged and stained for CD21 expression and viability. Cells were analyzed on an LSRII flow cytometer (BD) and evaluated with FlowJo v10 software (Tree Star).

3.4 Results

Antigen production

Nsp7 was expressed at high levels in *E. coli* Rosetta cells. Three hours after isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, nsp7 appeared to account for >50% of total cellular protein, and was homogeneous on an SDS-polyacrylamide gel following cobalt

metal affinity chromatography (Fig.5A). Purified nsp7 was highly antigenic and specifically bound by antibodies from PRRSV immune serum but not from PRRSV nonimmune serum (Fig 5B).

Antigen biotinylation

Quantitative biotinylation is required to form the tetrameric antigen complex that is key to the method. The goal is to achieve molar equivalence so that antigens contain only one biotin and minimal disruption of native antigenicity. Hence, biotin was titrated into purified nsp7 at molar ratios of 1:1 and 1.2:1 to nsp7. The labeling results were evaluated with a streptavidin quenching western blot. The efficiency of biotin labelling was determined by first incubating a fixed concentration, 2 uM, of biotinylated nsp7 with titrated amounts of SA-PE from a saturating excess to insufficient. The reactions were electrophoresed on a non-denaturing native gel, transferred to a nitrocellulose membrane, and then probed with the detecting reagent, Alexa Fluor 680 conjugated SA (SA-AF680). As the concentration of SA-PE in the mixture decreased, unbound biotinylated nsp7 was permitted to bind to the detecting reagent, SA-AF680, resulting in the fluorescent exposure of bands on the western blot.

At a biotin to nsp7 molar ratio of 1:1, we observed the appearance of western blot bands at low concentrations of SA-PE (0.031-0.062 µM), indicating that less than 25% of nsp7 protein was biotinylated (Fig. 6). At a 1.2:1 ratio, a band was clearly reduced at 0.25 µM and substantially quenched at 0.5 µM (Fig. 6). SA-PE has 4 binding sites for biotin. Thus, the failure to quench 2 uM of biotinylated protein at 0.125 µM of SA-PE indicates that greater than 25% of nsp7 was labeled with 1 molecule of biotin.

Antigen tetramer formation and validation

Biotinylated nsp7 was mixed with SA-PE, as described in the methods, to fully arm the tetramer. Unbiotinylated nsp7 was removed by size exclusion ultrafiltration, and the tetramer was analyzed by denaturing SDS gel electrophoresis. As shown in Figure 5, lane 4, nsp7 (30 kDa) was present. Interestingly, PE (~250,000 kDa) and SA (~55 kDa) do not run on SDS-PAGE gels at their native molecular weights of 240 kDa and 53 kDa, respectively (Fig. 5A). Rather, PE dissociates into three subunits, α, β, and γ with reported molecular weights of 18.3, 19.6, and 33.7 kDa, respectively (Liu et al., 2005; Munier et al., 2015). Streptavidin is a homotetramer which dissociates into four monomers that migrate at ~12 kDa, as shown in Figure 5, lane 5 (Lim et al., 2013).

To confirm that the nsp7-tetramer retained specific immunoreactivity, the tetramer was incubated with PRRSV antibody positive and negative serum and anti-PE microbeads, magnetically selected on an LS column, and washed to remove unbound antibody. After elution, centrifugation, and washing of the magnetic bead pellet containing bound antibodies, the mixture was resuspended in 5% non-fat dry milk and tested in an anti-porcine IgG capture ELISA for the presence of immunoglobulin bound to the nsp7 tetramer. Evaluation of 5 naïve and 5 immune animals showed nsp7 retained specific immunoreactivity for IgG after biotinylation and tetramer formation (Fig 7).

Antigen specific B cell identification

Nsp7-tetramer was evaluated for specific enumeration of antigen-specific memory B cells using tetramer pulldown followed by FACS analysis. For tetramer pulldown, 80 million live tracheobronchial lymph node cells from a PRRSV immune pig, and from one PRRSV naïve animal of the same age, were thawed from liquid nitrogen, stained with the decoy tetramer, SA-PE conjugated to AF647, to detect B cells that bind common components of the tetramer, such as SA or PE, but not nsp7. Next, cells were stained with the nsp7-tetramer, followed by anti-PE microbeads, which bound PE on both the decoy and the nsp7 tetramer. Following positive magnetic enrichment for PE, cells were stained for viability and CD21, a component of the B cell antigen receptor. Eluted, enriched cells from each animal were then resuspended in 425 ul of 4% paraformaldehyde and run on an LSRII flow cytometer for 8 minutes at identical flow rates.

Flow cytometry results were gated and evaluated as shown in Figure 8. Analysis of the total lymphocyte populations before pulldown showed that there were more lymphocytes in the tracheobronchial lymph node of the immune animal than the naïve animal (Fig. 8A). After gating for lymphocytes, single cells, and live B cells, the immune animal also had a large population of B cells bound to the nsp7 tetramer whereas the naïve animal had a much smaller number of tetramer-bound cells (Fig. 8B). Additionally, there were many more nsp7-specific, live B cells in the tracheobronchial lymph node of the immune animal (Fig. 8C). When controlling for the number of live B cells within the tissue, there was an approximately six-fold difference in the prevalence of nsp7-specific live B cells between immune and naïve animals (Fig. 8D).

Tetramer pulldown effects on live B cell numbers

Tetramer pulldowns are designed to highly enrich antigen-specific cells by selective, magnetic retention. During evaluation of its effectiveness, we noticed that there was a pronounced difference in nonspecific selected cells following pulldown, as well as in nsp7-tetramer-specific B cells, between the naïve and immune populations (Fig. 8B). We also noted here, and in additional experiments, that total lymphocytes and B cell frequencies were substantially higher in lymphoid tissues from immune animals (Fig. 8A).

To determine if immune status affected the quality of tetramer pulldown enrichment, frozen splenocytes from 30 naïve and immune animals were thawed and analyzed with nsp7-tetramer pulldown. Comparison of starting and eluted live B cell numbers showed a highly significant correlation regardless of initial starting cell number (Fig 9A). Generally, a 1000-fold enrichment was achieved by the tetramer pulldown (Fig 9B).

3.5 Discussion

Limitations and restrictions on the prophylactic and therapeutic use of antibiotics for disease control, as well as emerging and re-emerging viral diseases, in food animal species provide a compelling need for immunological means of disease prevention and intervention. Vaccination is a highly effective immune countermeasure, but effective vaccines have proven difficult to develop against a number of important veterinary animal diseases. In swine alone, a wide range of bacterial pathogens, including, but not

limited to *Hemophilus parasuis*, various *Mycoplasma* spp., *Mycobacterium* spp., *Pasteurella* spp., *Brachyspira* spp., and *Bordetella brochiseptica*, are not effectively controlled with existing products. With respect to viral pathogens, PRRSV vaccines are incompletely effective, while porcine circovirus 2 vaccines are highly effective, but the mechanism of protection is not known and new variants that are emerging raise concerns about future protection.

Memory B and T lymphocytes are the key surveillance and effector cells of adaptive immunity which are responsible for vaccine efficacy. Reagents and tools that facilitate identification, monitoring and characterization of pathogen-specific memory lymphocytes will further a mechanistic understanding of vaccine efficacy and promote development of better products. Cell surface markers for memory B and T cells of veterinary species are sparse and, perhaps, incompletely characterized, and do not identify antigen- or pathogen-specific responses. However, tetramers are highly specific, sensitive, and biased toward detection of high affinity IgG expressed on the surface of memory B cells. In a reversal of typical approaches to development of immune cell markers, B cell tetramers provide a route to identification and isolation of memory B cells that are then analyzed for markers of memory.

Here, development of tetramers for pathogen-specific memory B cells is described, using PRRSV in swine as a relevant example. The porcine memory immune response to PRRSV is not well characterized, resulting in a major gap in knowledge about immunological resistance to genetically diverse viruses. Previous investigations into the porcine anti-PRRSV memory immune response have utilized ELISPOT, an effective assay for enumerating antibody secreting cells (Mulupuri et al., 2008; Rahe and

Murtaugh, 2017b). However, ELISPOT requires laborious cell culture, various conditions to differentiate plasma cells from memory cells, and is imprecise due to cell proliferation and differentiation that occurs during culture (Rahe and Murtaugh, 2017b). Tetramers avoid these problems by their ability to bind high affinity memory B cells directly isolated from animals or following recovery from frozen storage. In addition, they enable substantial characterization of antigen-specific B cell-mediated immune protection.

The creation of an effective tetramer to investigate the role of memory B cells in veterinary immunology and vaccinology starts with the production of a conserved, pure, highly immunogenic protein that is soluble in physiologically compatible solutions. In the case of PRRSV, nsp7 is known to be highly conserved among PRRSV-2 variants (2). Fortuitously, it is readily expressed at high levels in soluble form in *E. coli* and retains solubility after purification.

The appropriate quantitative biotinylation of nsp7 is arguably the most important aspect of tetramer manufacturing. While the reaction itself is straightforward, the desired ratio of biotin to nsp7, i.e. one biotin per molecule of protein, requires careful titration for each labeling reaction. Heavily biotinylated proteins have reduced immunoreactivity, whereas low levels waste valuable reagent. Under the conditions of our laboratory, a biotin:nsp7 ratio of 1.2:1 yielded an nsp7 product in the desired ratio range of 0.25-0.75 biotin per protein molecule. Following confirmation of the biotinylated nsp7 concentration and the removal of unbound biotin, the tetramer was fully armed by incubating biotinylated protein with the streptavidin-PE at a 4:1 molar ratio for 30 minutes at room temperature, after which remaining free nsp7 and unbound biotinylated nsp7 was removed by size exclusion centrifugation.

B cell tetramer recognition of cognate antibodies by ELISA established that nsp7 antigenic function and specificity was retained in the tetramer. The tetramer was incubated with PRRSV naïve or immune serum, magnetically enriched to remove unbound antibody, and then evaluated in an anti-porcine IgG capture ELISA to detect antibodies bound to nsp7 on the tetramer. To our knowledge, this is the first description of this novel assay which showed that antibodies from PRRSV immune animals are able to specifically bind the tetramer, whereas antibodies from naïve animals showed only background reactivity. Tetramer incubation with lymph node cells from naïve and immune animals showed that it specifically recognized antigen-specific B cells.

The rarity of antigen-specific memory B cells in blood and lymphoid tissues, combined with the structural complexity of the tetramer, results in high nonspecific backgrounds with minimal ability to directly identify target cells. Thus, an enrichment step is needed to enrich for antigen specific B cells and distinguish specific from nonspecific. Magnetic enrichment, referred to as tetramer pulldown in the literature, increased the frequency of specific, target memory B cells by about 1000-fold, and enabled FACS separation of specific cells labeled with PE from nonspecific background cells labeled with PE and APC. Tetramer pulldown followed by FACS analysis enabled the detection of nsp7-specific B cells at frequency of about 1/100,000 total B cells.

The tetramer pulldown enrichment step may lead to confusion in FACS data presentation and interpretation. During routine FACS analysis cells are gated sequentially for lymphocytes, single cells, live B cells, and then finally for nsp7-specific cells. The final flow plot, as shown in Figure 4, displays the decoy and nsp7 tetramer antigen specificity of all live B cells in the FACS sample. Hence, the nsp7-specific gated population shows

the percentage of nsp7-specific live B cells in the sample after tetramer pulldown. The remaining, non-gated cells are indicative of the amount of background non-specificity inherent to the pulldown method. For meaningful interpretations of antigen-specific B cell abundance and the strength of immune responses, we express the tetramer-specific cell populations as a frequency of total B cells that enter the pulldown method, or of total B cells present in the starting lymphoid tissue, or as an abundance in the entire lymphoid tissue itself.

The strong correlation between the starting number of live B cells in tetramer pulldown and the eluted number, regardless of immune status, shows that the method did not introduce a selection bias due to sample size or immune status. For example, beginning a tetramer pulldown with approximately 6 million live B cells results in roughly 6000 live B cells in the eluted fraction. Therefore, the number of nsp7-specific B cells that is displayed in a final flow plot is representative of the starting condition. In practice, we seek to harvest and process entire lymphoid tissues, or determine the proportion of total tissue that is processed. The total number of live leukocytes in a tissue sample is recorded prior to freezing in liquid nitrogen. After thawing, we record the proportion of total tissue cells used for an experiment, assess viability, and proportion of B cells by staining with CD21. At the end of an experiment, data can be normalized to the starting number of live B cells, or per million live B cells, or to total number in the tissue. The process removes the inherent variability which enrichment introduces and results in a more easily interpreted result.

B cell tetramers have been used in mice and humans to characterize recognition of self and foreign antigens as well as the development of the humoral immune response and

memory response to innocuous antigens (Hamilton et al., 2015; Li et al., 2012; Morris et al., 2011; Taylor et al., 2015). This information has been profoundly important in the understanding of basic B cell ontogeny and memory development. In the pig and other veterinary species, the memory immune response to viral infection is currently a black box due to the lack of effective tools and reagents. Creation of the nsp7 tetramer will facilitate an in depth investigation into the characteristics and quality of the memory B cell response generated to PRRSV infection and vaccination. The methods are applicable to all animal species for which relevant antigens are described or can be identified and produced in soluble form.

3.6 Acknowledgements

We thank Justin Taylor and Marc Jenkins for introduction to the methodology and advice in implementation, and Paul Champoux at the University of Minnesota flow core facility for expert technical assistance. The research was supported by USDA NIFA grant 2015-06966, an NIH fellowship to MCR from the training grant T32 OD010993, and an AASV Foundation grant for KG.

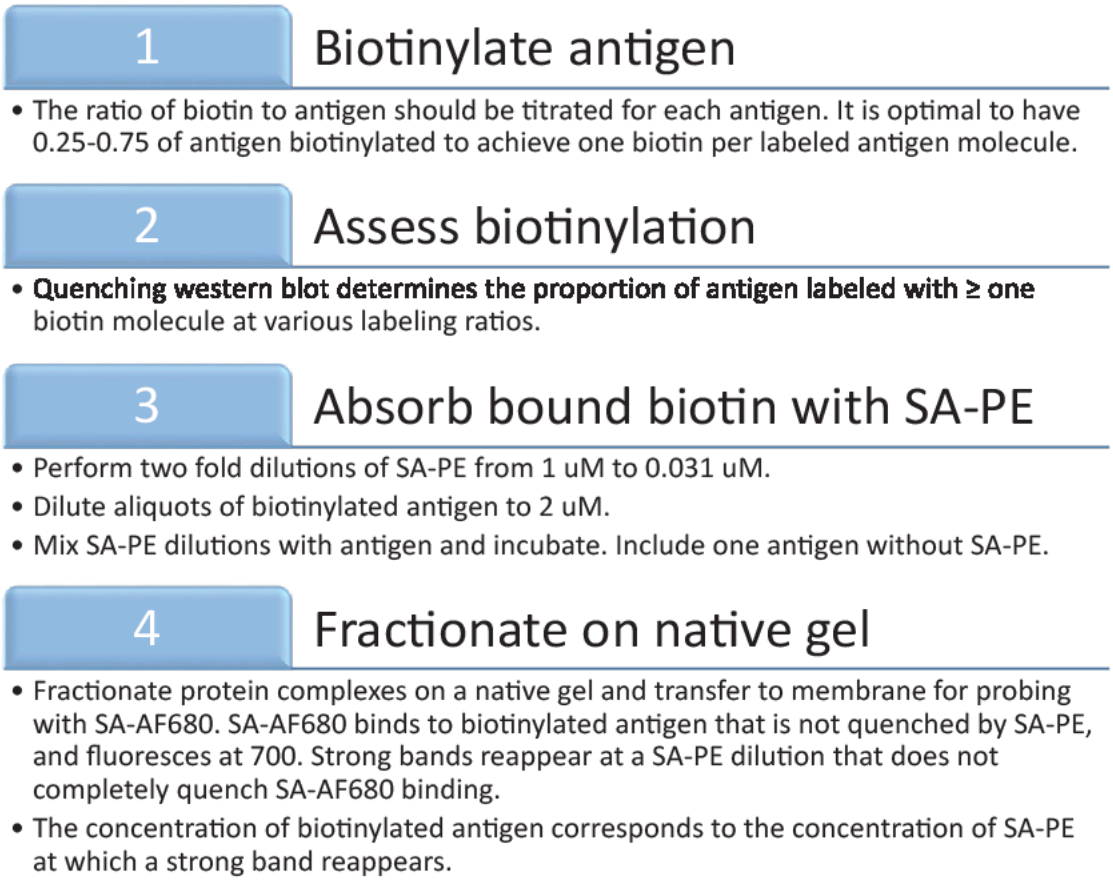

Figure 3-1 Workflow for the biotinylation of antigen

Figure 3.1 - Workflow for the biotinylation of antigen. The outlined process is used to first biotinylate antigen and then to evaluate the efficiency of biotinylation.

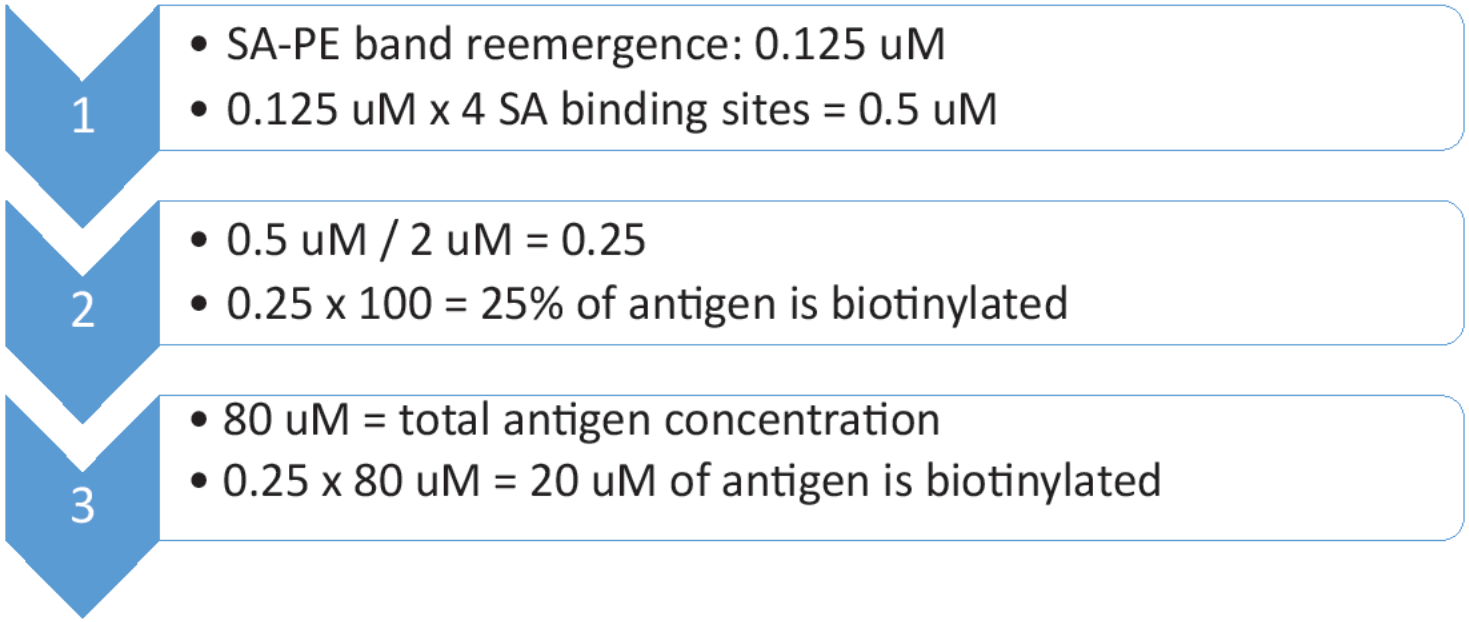

Figure 3-2 Calculation of biotinylated antigen concentration

Figure 3.2 - Calculation of biotinylated antigen concentration. Displayed equations were used to determine the concentration of antigen which was biotinylated.

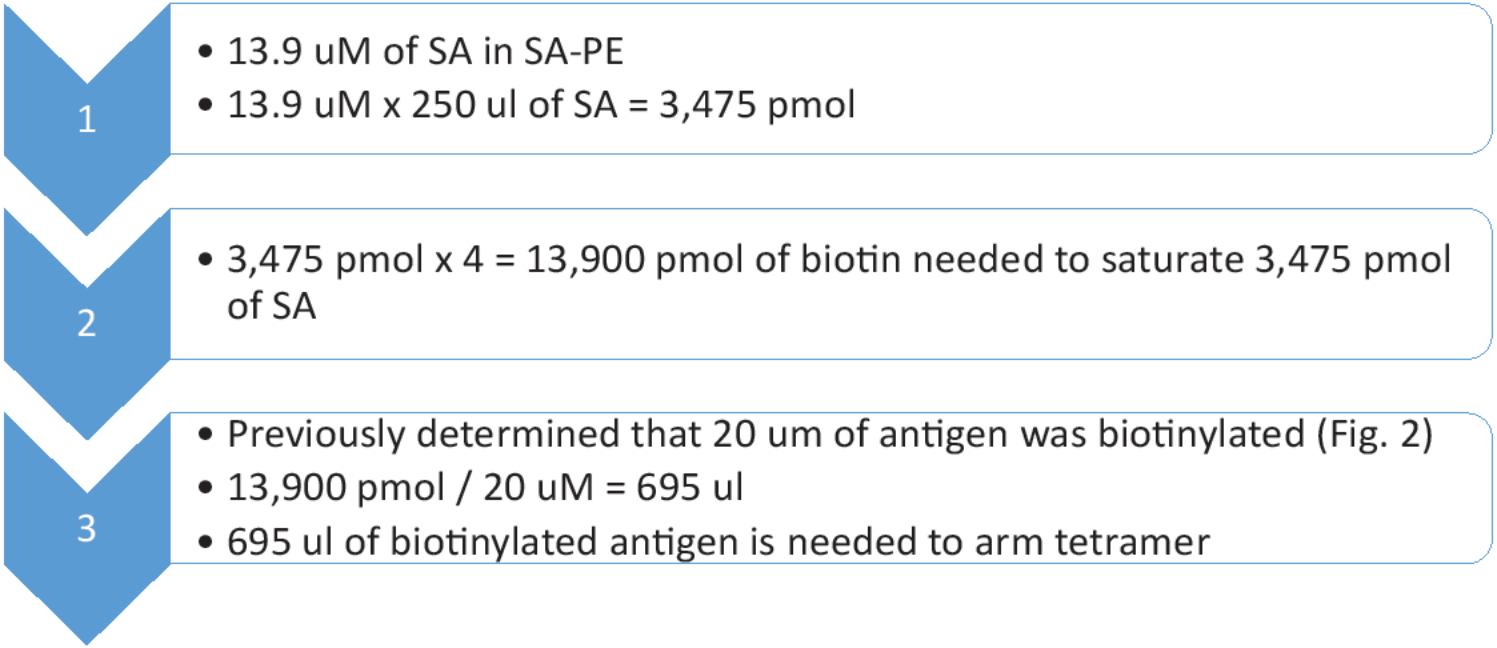

Figure 3-3 Calculation of biotinylated antigen required to fully arm tetramer

Figure 3.3 - Calculation of biotinylated antigen required to fully arm tetramer. Displayed equations were used to determine the moles of antigen necessary for full tetramer formation.

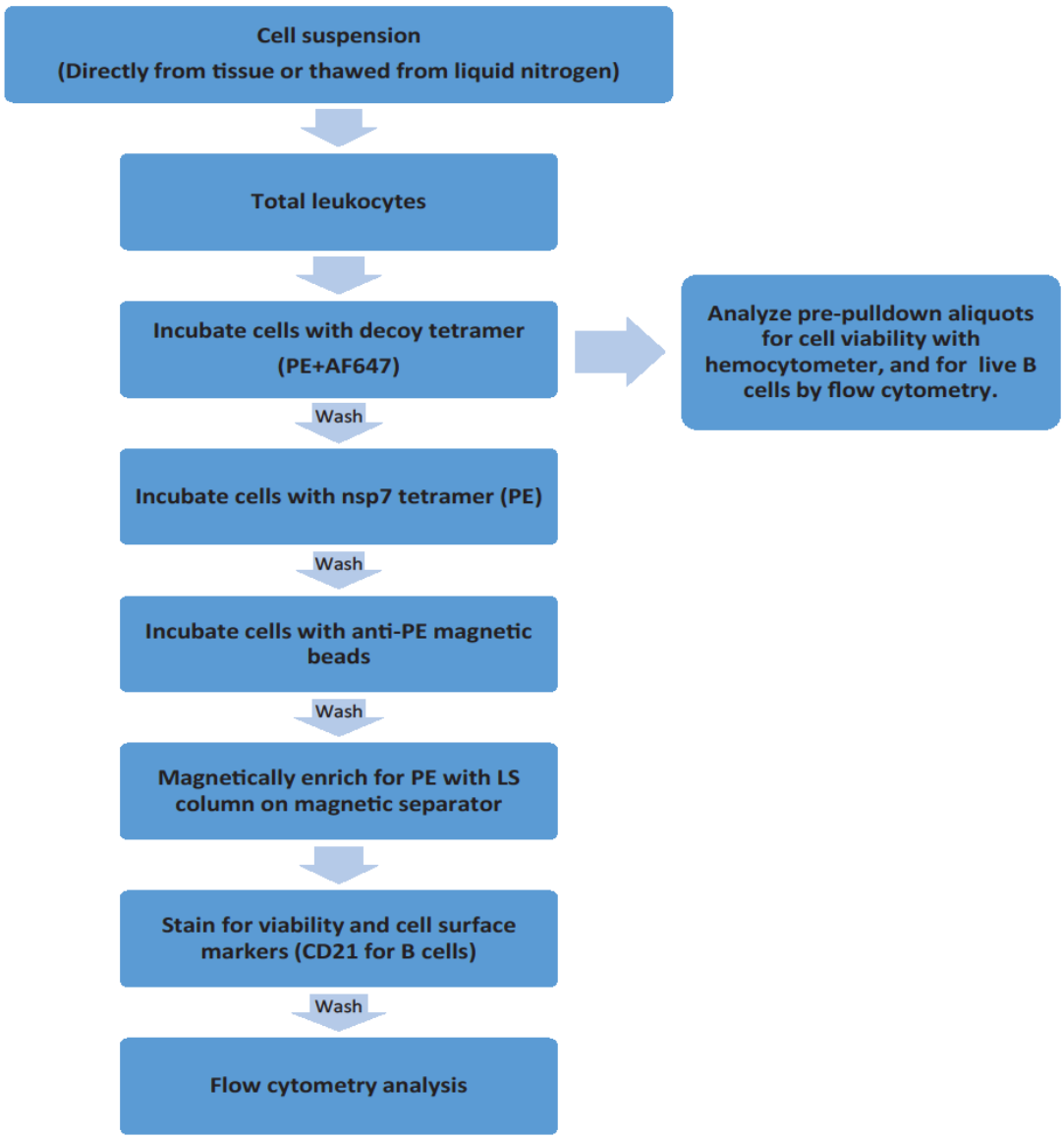

Figure 3-4 Tetramer pulldown workflow

Figure 3.4 - Tetramer pulldown workflow. The displayed protocol was used to identify and enumerate nsp7-specific live B cells as well as to determine the total number of starting live B cells.

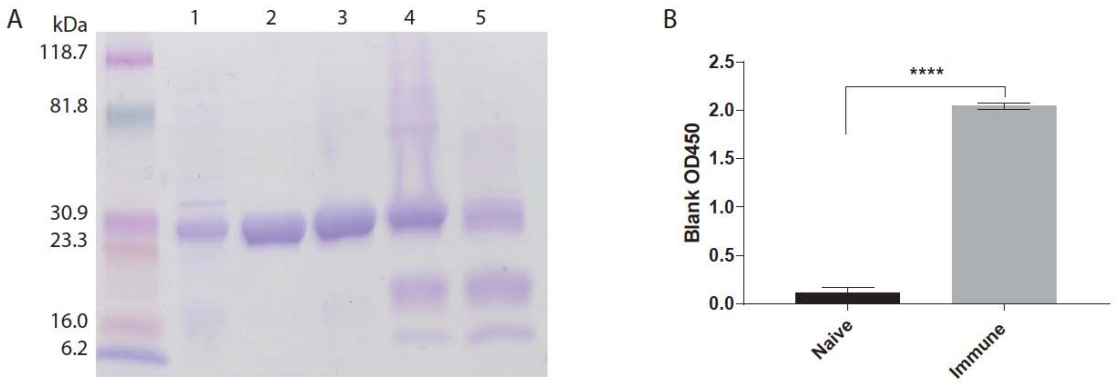

Figure 3-5 Recombinant nsp7 characterization

Figure 3.5 - Recombinant nsp7 characterization. (A) SDS denaturing PAGE gel showing fractions from nsp7 tetramer creation. Protein bands were visualized with Imperial Protein stain (Thermo Fisher Scientific). Lane 1. Whole cell lysate solubilized in SDS, with nsp7 migrating at approximately 30 kDa. Lane 2. Eight µg nsp7 eluted from a cobalt metal affinity column. Lane 3. Biotinylated recombinant nsp7. Lane 4. Nsp7 tetramer, including denatured streptavidin (SA) and phycoerythrin (PE). Lane 5. Reduced SA-PE. (B) Nsp7 indirect ELISA. Serum from 5 PRRSV naïve and 5 PRRSV immune animals was tested for immunoreactivity on microtiter plates coated with 100 ng nsp7. One representative experiment of three is displayed. Data are shown as mean ± SEM. Statistical differences of ***p< 0.001 were determined using the t-test.

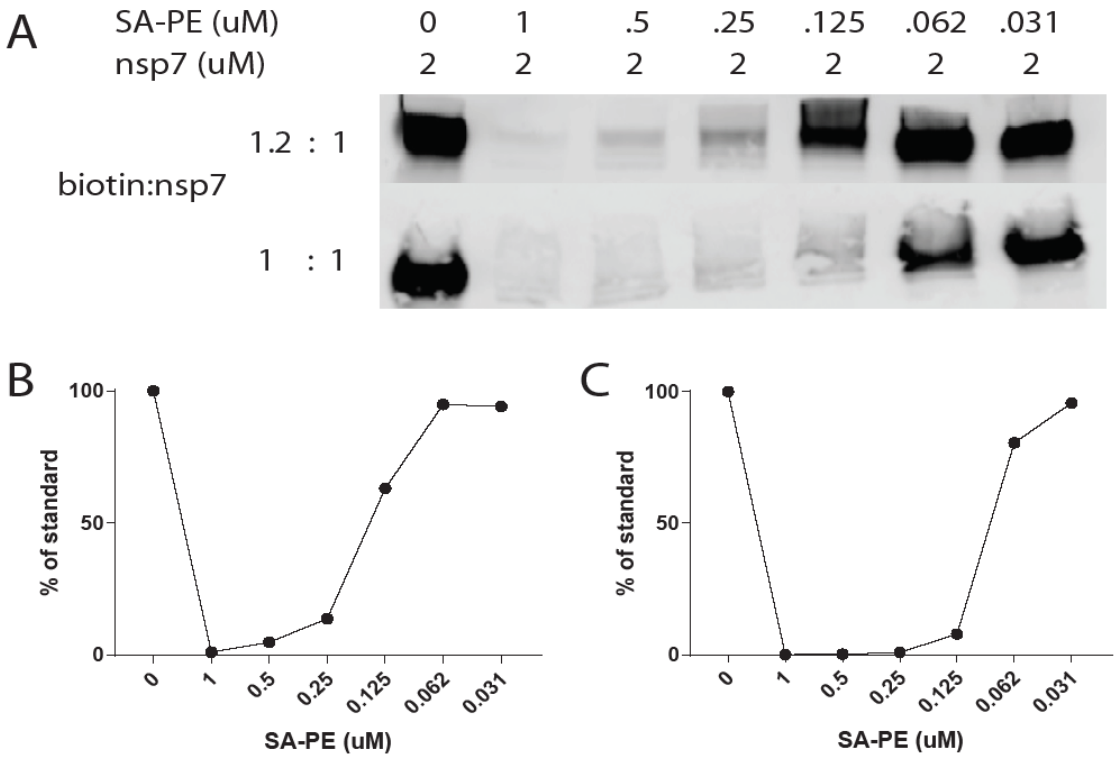

Figure 3-6 Analysis of biotinylation conditions for labeling antigen

Figure 3.6 - Analysis of biotinylation conditions for labeling antigen. (A) Nsp7 was biotinylated at 1:1 and 1:1.2 ratios of biotin to nsp7. Two uM of biotinylated-nsp7 were incubated with indicated amounts of SA-PE. Mixtures were then electrophoresed on a native gel and transferred to a nitrocellulose membrane. The membrane was probed with SA-AF680. The appearance and intensity of a western blot band reacting with SA-AF680 indicated the presence of biotinylated nsp7 that was not quenched by SA-PE. (B) Biotinylation efficiency at a ratio of 1.2 biotin to 1 nsp7. Band intensity is displayed as a percentage of the 0 uM SA-PE + 2 uM nsp7-biotin standard control. Reemergence of the band occurs at 0.125 uM SA-PE. (C) Biotinylation efficiency at a ratio of 1 biotin to 1 nsp7. Band reemergence is noted at 0.062 uM SA-PE.

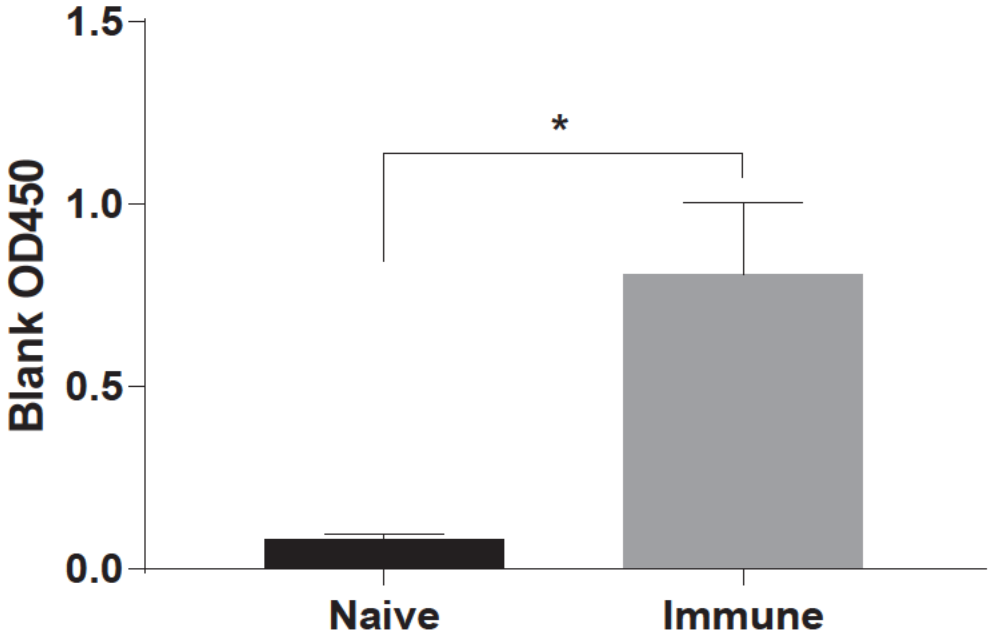

Figure 3-7 Specific immunoreactivity of nsp7 tetramer

Figure 3.7 - Specific immunoreactivity of nsp7 tetramer. Sera from 5 PRRSV naïve and 5 PRRSV immune pigs were incubated with nsp7 tetramer followed by anti-PE microbeads. Microbeads with bound antibodies were magnetically selected, washed and eluted. The eluted fraction was evaluated in an anti-porcine IgG capture ELISA. Data from one of three experiments is shown as mean ± SEM. Statistical differences of *p< 0.05 were determined using the t-test.

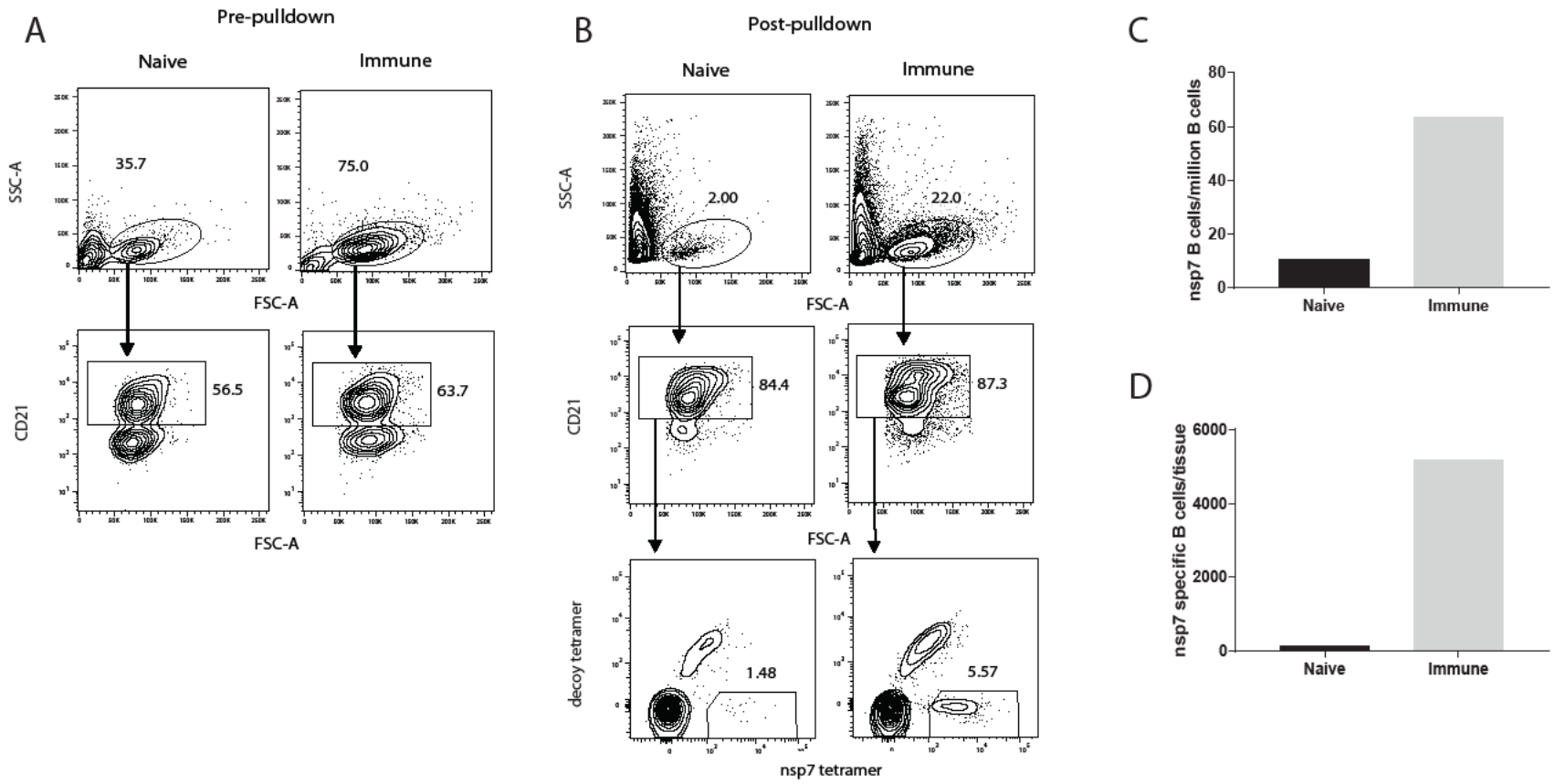

Figure 3-8 Nsp7 tetramer enumeration of antigen-specific B cells

Figure 3.8 - Nsp7 tetramer enumeration of antigen-specific B cells. Tracheobronchial lymph node cells from one immune and one naïve animal were thawed from liquid nitrogen counted, stained for viability and CD21 expression, and then analyzed by flow cytometry. (A) Forward and side scatter characteristics of live cells prior to pulldown, followed by CD21 staining of the gated population to identify the B cell population. (B) Cells were incubated with a decoy tetramer, nsp7 tetramer, and magnetic beads and then enriched by tetramer pulldown. Cells were stained for viability and CD21 expression and then analyzed with flow cytometry. The displayed gating strategy shows post-gating for live and single cells. Nsp7 tetramer gate on the bottom panels is from fluorescence minus one (FMO) controls. (C) Nsp7-specific cells were enumerated and plotted per tracheobronchial lymph node. (D) Nsp7-specific cells were enumerated and expressed as a proportion of starting live B cells.

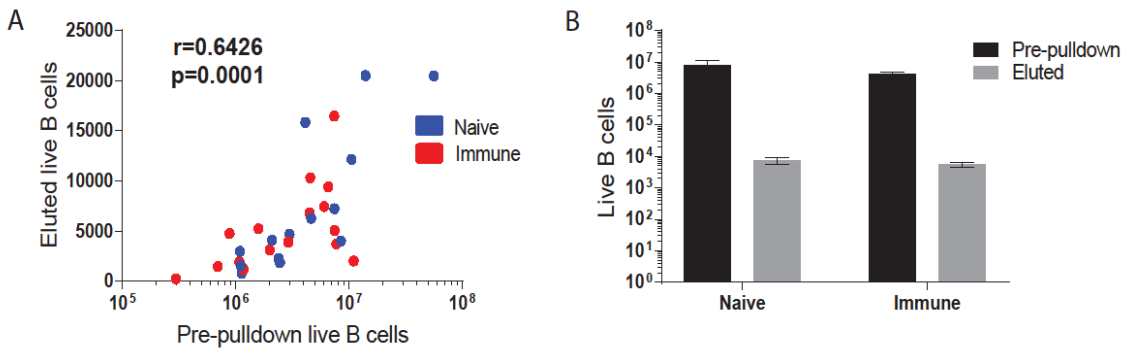

Figure 3-9 Live CD21+ B cells in splenocytes pre and post-tetramer pulldown

Figure 3.9 - Comparison of live CD21+ B cells in splenocytes pre and post-tetramer pulldown. (A) Tetramer pulldowns were carried out with various numbers of banked splenocytes from 30 PRRSV naïve and immune animals. Correlation analysis was performed using GraphPad Prism 7. (B) Mean±SEM of pre- and post-pulldown live B cells shown in panel A, indicating an approximately 1000-fold enrichment irrespective of starting cell number.

Chapter 4 - Regional specialization of PRRSV nsp7-specific porcine B cell kinetics post-vaccination

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4.1 Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most important pathogen of swine health and wellbeing worldwide largely due to an insufficient understanding of the adaptive immune response to infection. The memory and anamnestic response to infection are critical gaps in knowledge in PRRSV immunity. The lack of effective tools for the evaluation of the memory response previously hindered the ability to effectively characterize the porcine memory response to infection. However, the creation and validation of a PRRSV nsp7-specific B cell tetramer (four protein antigens linked together with a fluorescent marker) now facilitates the ability to detect very rare memory B cells and thus describe the memory response of the pig. Here, we describe the nsp7-specific B cell response in six key secondary lymphoid organs. PRRSV MLV viremia was detected at day7 and lasted until day 28. This resulted in anti-PRRSV IgG seroconversion starting at day 28 and increasing in intensity at day 56. An expanded population of nsp7-specific B cells was identified at day 28 in the inguinal, tracheobronchial, and mesenteric lymph nodes which contracted by day 56. An antigen specific B cell response was noted in the blood and spleen of immune animals at day 56. This definition of memory B cell kinetics to PRRSV will answer key questions involved in regional specialization of the immune response following intramuscular inoculation of PRRSV MLV.

4.2 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most important adversary of swine health and wellbeing worldwide. Discovered more than thirty years ago, there is still no effective intervention for inducing the development of a broadly protective immune response against the virus. While the reasons for this failure are multivariate, a large part of this deficiency lies in an incomplete understanding of fundamental aspects of the humoral immune response to PRRSV.

The humoral immune response to viral infection is made up of circulating antibodies and memory B cells. While antibodies are the effector mechanisms of this response, the induction of a memory response is necessary for the development of immune protection. Upon antigen recognition, memory B cells rapidly boost antibody titers with high affinity, isotype switched antibodies. Classical PRRSV immunity research has focused on the identification and characterization of neutralizing antibodies, generally considered to be the most important component of anti-viral immunity (Burton, 2002; Lopez et al., 2007; Osorio et al., 2002; Robinson et al., 2015). However, the memory B cell response to PRRSV has largely gone unstudied. As a result of this, there are significant gaps in knowledge which must be addressed if improved immunological interventions against PRRSV are to be developed.

PRRSV is known to infect monocytes/macrophages in specific anatomic regions of the pig (Duan et al., 1997a, b). Comparatively, there is anatomic regional variation in the antigen-specific B cell response to viral infection in the pig (Mulupuri et al., 2008; Yuan et al., 2001). However, previous research was performed only with ELISPOTs due to a dearth of tools and reagents for a sensitive, specific, and rapid study of PRRSV antigen

specific B cells. In addition, this prevents the downstream study of antigen specific cells. Fortunately, the creation of a PRRSV nsp7 B cell tetramer has now made an in depth investigation of the porcine antigen specific B cell response to viral infection possible.

The nsp7 B cell tetramer consists of four biotinylated nsp7 proteins linked together via a streptavidin core which is coupled to phycoerythrin, a bright fluorophore. Upon incubation with suspended B cells, this presents four opportunities for the surface immunoglobulin of B cells to bind the reagent. Once bound, there are three other antigens which may be bound by additional surface immunoglobulins of the B cell, resulting in a highly avid reagent. However, because nsp7-specific B cells within tissue are very rare, approximately 1 in 100,000 B cells, a magnetic bead enrichment step is necessary to concentrate and enumerate tetramer bound cells (Rahe and Murtaugh, 2017b).

Here, we investigate the nsp7-specific B cell dynamics in six secondary lymphoid organs of the pig up to 56 days after vaccination with a MLV PRRSV. Tissues were selected based on previous research identifying them as playing a significant role in PRRSV infection and immunity, as well as being readily identifiable and easy to be collected and processed. These findings will inform further investigations into the porcine antigen-specific B cell response to PRRSV, such as the presence and magnitude of an anamnestic response upon challenge. Finally, we present a model which can be employed for in depth investigations into the adaptive immune response for any veterinary species and pathogen for which the B cell response warrants investigation.

4.3 Materials and Methods

Animal study and tissue collection

The animal study was funded by Boehringer Ingelheim, approved by Veterinary Resources, Inc. (Ames, IA) animal care and use committee and carried out under their purview. Twenty pigs of approximately three weeks of age were used for this study. On day 0, 15 animals were inoculated with 2 ml of Ingelvac PRRSV MLV, 5 animals remained PRRSV naive and were housed in a separate group from vaccinated animals. On days 0, 7, 14, 28, and 56 three animals from the vaccinated group and one animal from the unvaccinated group were sacrificed and had the following tissues collected: 100 mls of blood, spleen, superficial inguinal lymph node (ILN), mesenteric lymph node (MLN), tracheobronchial lymph node (TBLN), and tonsil. Tissues were sectioned, placed in tissue culture media (complete RPMI 1640 with L-glutamine, 5% fetal bovine serum (FBS), 10 mM HEPES pH 7.2, 1% non-essential amino acids, 1% sodium pyruvate, and 20 ug/ml gentamycin) and shipped overnight to the Murtaugh lab.

Upon receipt, whole blood was diluted 1:2 with 1x PBS. Diluted blood was then slowly layered over lymphocyte separation media (LSM) at a 1:3 dilution in a 50 ml conical tube. Samples were centrifuged at 2,000 RPM at 20°C for 30 minutes with the brake off. Following centrifugation, the PBMC layer was aspirated, transferred to a new tube, and washed in 1x PBS. Following centrifugation at 1,400 RPM for 10 minutes, pelleted cells were resuspended in ACK lysis buffer (Lonza) at a dilution of 1:6 pellet to ACK to lyse remaining red blood cells. Samples were incubated, with gentle rocking, for 10 minutes. ACK was then diluted 1:2 with complete RPMI and centrifuged at 1,400 RPM for 10 minutes. Pelleted cells were resuspended in complete RPMI and counted with a

hemocytometer. Samples were centrifuged at 1,400 RPM for 10 minutes and then resuspended in freezing media (50% FBS, 40% complete RPMI, 10% DMSO), aliquoted into 2 ml freezing tubes (20-40 million cells/tube), placed in -80°C overnight and then transferred to liquid nitrogen storage the next day. Fifteen ml of serum was also collected per sacrificed animal.

Solid tissue was processed as previously described with the following modifications (Rahe and Murtaugh, 2017b). Lymph nodes and tonsil were scored with a sterile razor blade prior to cellular dissociation with the plunger of a syringe. Following ACK lysis resuspension and incubation, ACK lysis buffer was diluted 1:2 in complete RPMI + 5% FBS media. Cells were frozen at between 20-40 million cells/tube depending upon the cellular yield from each tissue.

nsp7 ELISA

Recombinant nsp7 ELISA was performed as previously described in chapter 3.

nsp7 tetramer pulldown

Nsp7 tetramer pulldown was performed as previously described in chapter 3.

Viral RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

Viral RNA was isolated from 140 ul of serum using a QIAmp Viral RNA Mini kit (Qiagen) and eluted with 50 ul of RNAse free water. Ten ul of eluted RNA was then used to synthesize complementary DNA (cDNA) with the High Capacity cDNA synthesis kit

(Applied Biosystems). RNA fragments were amplified with the following PRRSV-2 specific primers, forward ORF7L (aaccacgcatttgtcgtc) reverse ORF6R2 (tggcacagctgattgactgg). All PCR reactions were performed in a total volume of 20 ul (5 ul forward and reverse primers, 10 ul SYBR green master mix, 5 ul cDNA) and run on a Stratagene Mx3000P thermal cycler (Agilent). All samples were run in triplicate. Viral RNA was quantified by comparison to a standard curve.

4.4 Results

PRRSV MLV viremia

Fifteen animals were inoculated at day 0 with Ingelvac PRRSV MLV. Peak viremia was detected at 7 days post-vaccination (Fig 4.1). Two of three inoculated animals were positive at day 14, though positive animals had a two-fold reduction in viral copies per ml compared to viremia levels on day 7. Again, two of three inoculated animals were positive at day 28. However, these animals displayed viremia levels one log less than those observed at day 7 but one log greater than detected levels from animals at day 14.

nsp7 seroconversion

Seroconversion to nsp7 was evaluated with an nsp7-specific indirect ELISA. Both inoculated and naïve animals were negative from day 0 through day 14 of the study (Fig 4.2). Two of three vaccinated animals were seropositive on day 28. The lone seronegative

vaccinated animals was also negative for viremia at this time. On day 56, all three vaccinated animals were seropositive.

Blood and spleen nsp7-specific B cell response

Blood and sections of spleen were collected from one naïve and three sacrificed animals at designated time points of the study. PBMCs and splenocytes were isolated from these tissues and frozen for later analysis. After thawing, five percent of cells were aliquoted for enumeration of live cells as well as staining for viability and B cell analysis with flow cytometry, to determine the total number of starting live B cells prior to tetramer pulldown. The remaining cells were incubated first with a decoy tetramer, followed by the nsp7 tetramer, and then anti-phycoerythrin microbeads with cell washes occurring between incubations. Magnetic microbeads bind to both the decoy and the nsp7 tetramer, following passage over an LS column within a magnetic separator tetramer bound cells are retained. Cells are then eluted off of the column and stained for viability and CD21, a marker for mature B cells, expression (Sinkora et al., 2013). Finally, cells are evaluated with flow cytometry to enumerate the total number of nsp7-specific cells within the sample. This number is then divided by the total number of starting B cells and multiplied by one million to determine the number of nsp7-specific cells per million B cells. The flow cytometry and gating strategy used for analysis are displayed in figure 4.3.

The dynamic response of nsp7-specific B cells to PRRSV MLV vaccination within the PBMC and splenocyte populations are strikingly similar in timing and are comparable in magnitude. The nsp7-specific response B cell response within the PBMC population of vaccinated animals is indiscernible from that of naïve animals for the first 28 days post-

vaccination (Fig 4.4A). However, at day 56 there is a clear difference between the immune and naïve response as the proportion of nsp7-specific B cells continues to rise in immune animals. Remarkably, the naïve proportion of nsp7-specific B cells continues to increase from day 0 until day 28 but then recedes sharply at day 56. Increasing the power of the study would have been helpful for determining if naïve animals on day 28 or 56 are outliers, if any.

There was an increasing proportion of nsp7-specific B cells in the spleen for both naïve and immune animals from day 0 until day 14 (Fig 4.4B). However, from day 28 until day 56 there is no increase in the proportion of nsp7-specific B cells in naïve animals; whereas, in immune animals the proportion of nsp7-specific B cells doubles from 20 per million cells to 40 per million cells. Notably, the nsp7-specific B cell response in both the spleen and PBMCs is a gradual expansion in number with no contraction.

TBLN and Tonsil nsp7-specific B cell response

The tracheobronchial lymph node and the tonsil were evaluated for their nsp7 specific B cell response due to their importance for respiratory infections as well as their previously identified regional association with PRRSV infection (Haynes et al., 1997; Rossow et al., 1996). As expected, on days 0-7 the nsp7-specific B cell response in the TBLN represented only naïve lymphocyte recognition (Fig 4.5A). However, from day 14-28 there is a marked expansion of nsp7-specific B cells with a contraction of this population near naïve levels by day 56.

The antigen specific lymphocyte response to vaccination in the tonsil was relatively unremarkable. On day 28, there was one vaccinated animal which showed a markedly expanded population of nsp7-specific B cells (Fig 4.5B). Though, there was no difference in numbers between immune and naïve animals at all other evaluated time points.

ILN nsp7-specific B cell response

Analysis of the number of nsp7 specific B cells/million B cells displayed no remarkable difference between vaccinated and naïve animals with the possible exception of day 56 (Fig 4.6A). However, evaluation of the total number of nsp7-specific B cells in the inguinal lymph nodes which were processed and evaluated displayed an expansion of antigen specific B cells from days 14-28 (Fig 4.6B). This expanded population of B cells then contracted between day 28 and day 56 as was previously observed in the TBLN (Fig 4.5A). The slight increase in the total number of nsp7-specific naïve B cells in our naïve animal from day 28-56 was attributed to growth of the animals, the resulting increase in size of the ILN, and the consequential increase in the number of naïve lymphocytes capable of nsp7 recognition.

MLN nsp7-specific B cell response

Evaluation of the kinetic response of nsp7-specific B cells in the MLN showed a remarkably similar expansion and contraction of antigen specific cells in the immune animals as compared to the naïve animal. Between day 14 and 28 there was nearly a three-fold expansion in the number of nsp7-specific B cells per million B cells in the

MLN (Fig 4.7). This increase in antigen specific cells then shrunk between day 28 and 56.

4.5 Discussion

The humoral immune response to viral infection is driven by two effectors: antibodies and memory B cells. Antibodies exist to neutralize and/or clear pathogens while memory B cells work to rapidly boost antibody titers following antigen recognition. In the case of PRRSV, the vast majority of research has focused on characterizing the antibody response to infection. However, the memory B cell response has scantly been investigated. As a result of this, there are many basic questions which must first be answered for the development of a foundational knowledge upon which more advanced investigations may be built. With the use of the novel nsp7 B cell tetramer, we asked if there was regional variation in the antigen specific immune response to PRRSV vaccination. We sought to answer this question by identifying the kinetics of the nsp7-specific B cell response to PRRSV MLV vaccination in five PRRSV important secondary lymphoid organs and blood.

The ability to detect the expansion and contraction of antigen specific B cell populations was dependent upon a modified live virus which would infect and stimulate the innate immune response. Here, the modified live virus was able to actively replicate and sustain itself within the host until sometime between 28 to 56 days post-vaccination (Fig 4.1). While day 28 was the last day that viral RNA was detected in serum, this was also the

first day that anti-nsp7 IgG was detected. The timing of anti-PRRSV antibody emergence was in concordance with previous research identifying nsp7-specific IgG in animals inoculated with a strain of PRRSV-2 (Brown et al., 2009).

This data corresponded closely with the rise of nsp7-specific B cell populations within the isolated secondary lymphoid organs. For all of the studied lymph nodes, day 28 was the high point for nsp7-specific B cells within the total B cell population. The identified rapid proliferation was indicative of germinal center formation. By day 56, the number of nsp7-specific B cells had contracted within the lymph nodes, but was still on the rise in the spleen as well as the B cell population of the blood. The reason for the decline of these antigen specific populations in the lymph node can be attributed to the disappearance of germinal centers. However, the rapid expansion of nsp7-specific B cells in the spleen and blood during the same time is less clear. Though, this accounts for the large increase in anti-nsp7 IgG OD450 values between day 28 and day 56. It is possible that germinal centers in the spleen are maintained longer than in lymph nodes due to the viremic nature of the pathogen. The large increase in nsp7-specific B cells in the blood may be explained by the migration of antigen specific cells out of the lymph nodes, and spleen, and into the blood where these cells would be more likely to contact nsp7 in the future.

Initial analysis of the nsp7-specific B cell response in the inguinal lymph nodes of vaccinated animals was remarkable due to the paucity of a proliferative response at day 28. Analyzed as the number of nsp7-specific B cells divided by the total number of starting B cells, immune animals showed values consistent with our naïve control animals. This was noteworthy as we had observed enlarged inguinal lymph nodes in

immune animals, on day 28, at the time of processing. Additionally, the inguinal lymph node is known to be highly reactive in PRRSV immunity (Mulupuri et al., 2008; Xiao et al., 2004). Examination of the raw data showed that there were a large number of nsp7-specific B cells in two vaccinated animals which had seroconverted at day 28. Therefore, re-analysis of the data by looking at all of the nsp7-specific B cells which had been identified out of the entire inguinal lymph node led to a more accurate representation of the immune response for this lymph node. Possibly the proliferation of other PRRSV antigens in the inguinal lymph node led to the diluting out of nsp7-specific B cells in our initial analysis. Why this seems to have only occurred in the inguinal lymph node is unclear and warrants further investigation.

The observed nsp7-specific B cell response in the mesenteric lymph nodes of vaccinated animals was remarkable. This area of the body is thought to primarily respond to antigens from the intestines. However, we observed a clear nsp7-specific B cell response following vaccination. This corresponds with previous studies which have reported that PRRSV can induce a lymphocytic response in the lymphoid tissue of the intestines (Halbur et al., 1995; Xiao et al., 2004). The identified response in the mesenteric lymph nodes is most likely due to systemic blood flow and the viremic nature of the pathogen. Future investigations should determine if stimulation of the mesenteric lymph nodes by PRRSV results in anti-PRRSV IgA secretion into the intestinal tract. If so, this could be very important for the development of a mucosal immune response against enteric pathogens of the pig.

The antigen specific immune response in the tonsil was surprisingly unremarkable. On day 28, only one vaccinated animal showed a positive nsp7-specific response. The reason

for this failure is likely to do with the location of the immunization, as the intramuscular injection in the neck bypasses the tonsil. Furthermore, blood flow to the tonsil is minimal thus negating the ability of the viremia to induce a strong response. Comparison of this response in the tonsil with intranasal inoculation would be interesting as this should induce a strong tonsillar immune response.

The immune response in the tracheobronchial lymph node was as expected. This lymph node is important for drainage of the lungs and bronchi. Therefore, this is a prime location for antigen drainage and presentation in PRRSV infection. As a result of this, there was a strong expansion of nsp7-specific B cells on day 28 followed by an expected contraction at day 56.

While this study is important for laying the groundwork of antigen specific B cell kinetics, this study was hindered by the necessity for cross-sectional sacrifice of animals for tissue collection. Preferably, this research would have been carried out with a larger sample size for both our immune and naïve populations and one would be able to track the same animals longitudinally. However, the location and physiological necessity of the selected tissues in this study made longitudinal tissue collection impossible. Therefore, we were relegated to studying individual animals at selected time points while controlling for as many factors, such as age, inoculation, and environment, as possible.

The big picture focus of this work was to identify the memory B cell response to vaccination. However, we were impeded by the lack of a validated cell surface protein known to identify memory porcine B cells. Therefore, while we focused on the identification of antigen-specific cells, future work will build upon the developed,

reagents, methods, and findings to identify and validate a marker of the porcine memory B cell. The ability to identify to antigen specific cells makes this task possible.

First, nsp7-specific B cells from vaccinated animals should be FACS sorted at least 56 days post-vaccination, along with nsp7-specific B cells from naïve animals. RNA sequencing and analysis of nsp7-specific B cells from both immune and naïve animals will highlight differences in gene expression as well as identify key genes which may serve as memory B cell markers. The translation of these key genes' RNA into DNA will need to be confirmed with either mass spectrometry or flow cytometry, if a monocloncal antibody already exists to the potential proteins of interest. Finally, B cells will need to be isolated based on the expression of both CD21 as well as the surface protein of interest and evaluated for memory activity with a functional assay, such as ELISPOT. The identification of a surface protein marker by which to identify and analyze the memory B cell of the pig will be a leap forward in terms of porcine immunity. More importantly, it will allow for a more in depth investigation into the porcine adaptive immune response, not only for PRRSV, but for any pathogen which threatens swine health and wellbeing.

In conclusion, this study has identified that there are regional differences in the development of an nsp7-specific B cell response to vaccination with a PRRSV MLV. Antigen-specific B cell populations in the ILN, MLN, and TBLN expand quickly and robustly within 28 days post-vaccination, but these populations also contract shortly thereafter. Conversely, while the nsp7-specific B cell populations of the spleen and blood also proliferate they do not shrink as quickly as the lymph nodes. What role these differences play in PRRSV immunity warrants further investigation. The ability to identify antigen specific cells at different time points in the immune response should lead

to an enhanced understanding of PRRSV immunity as well as a more complete characterization of cellular phenotypes within the porcine adaptive immune response.

4.5 Acknowledgements

We thank Diem Gray and Emily Tosky for their assistance and expertise with animal tissue processing. The research was supported by USDA NIFA grant 2015-06966 and an NIH fellowship to MCR from the training grant T32 OD010993.

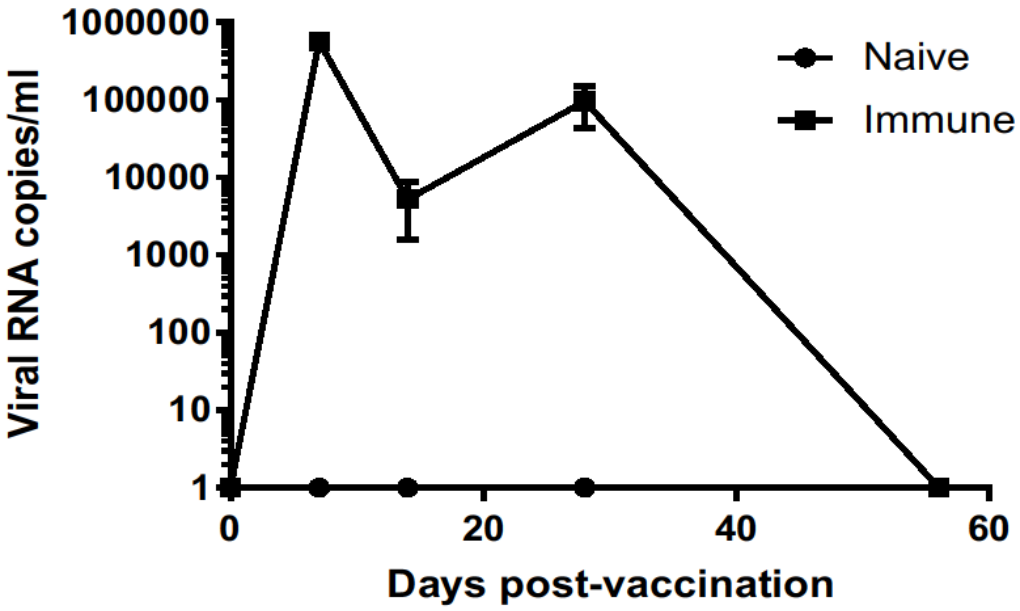

Figure 4-1 PRRSV MLV viremia

Figure 4.1 – Ingelvac PRRSV MLV viremia. PRRSV quantification from serum of immune and naïve animals over the course of 5 time points (0, 7, 14, 28, and 56 days post-vaccination). Each time point corresponds to three immune animals and one naïve animal which were bled and then sacrificed. The immune group data are represented as mean +/- SEM.

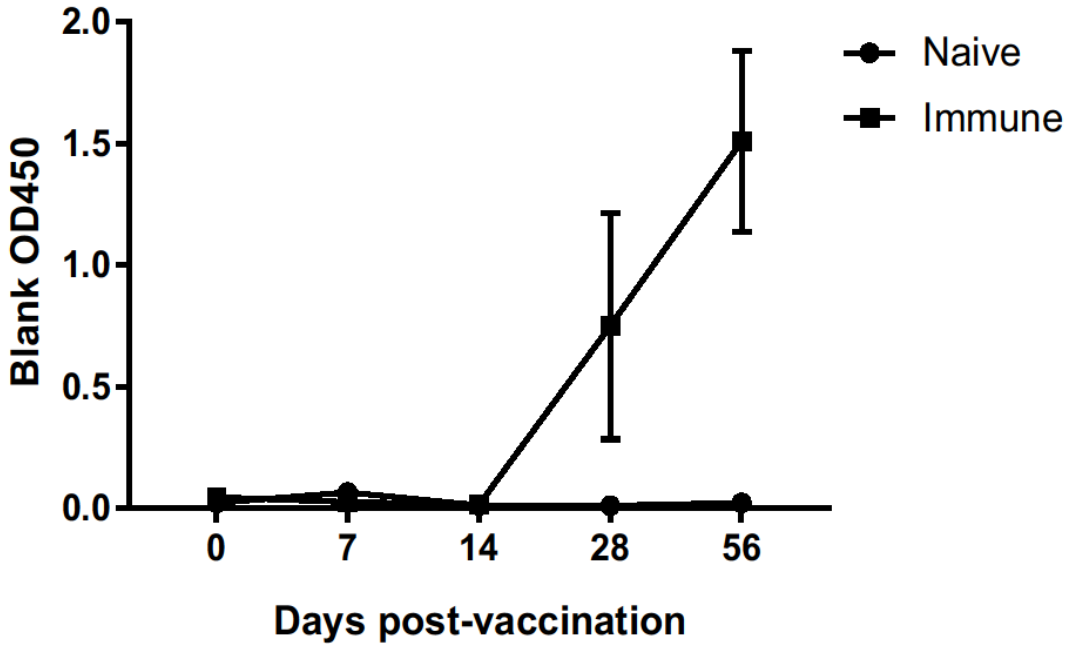

Figure 4-2 nsp7 seroconversion to PRRSV MLV

Figure 4.2 – nsp7 seroconversion to PRRSV MLV. Nsp7 indirect ELISA results for five time points (0, 7, 14, 28, and 56 days post-vaccination). Each time point corresponds to three immune animals and one naïve animal which were bled and then sacrificed. The immune group data are represented as mean +/- SEM.

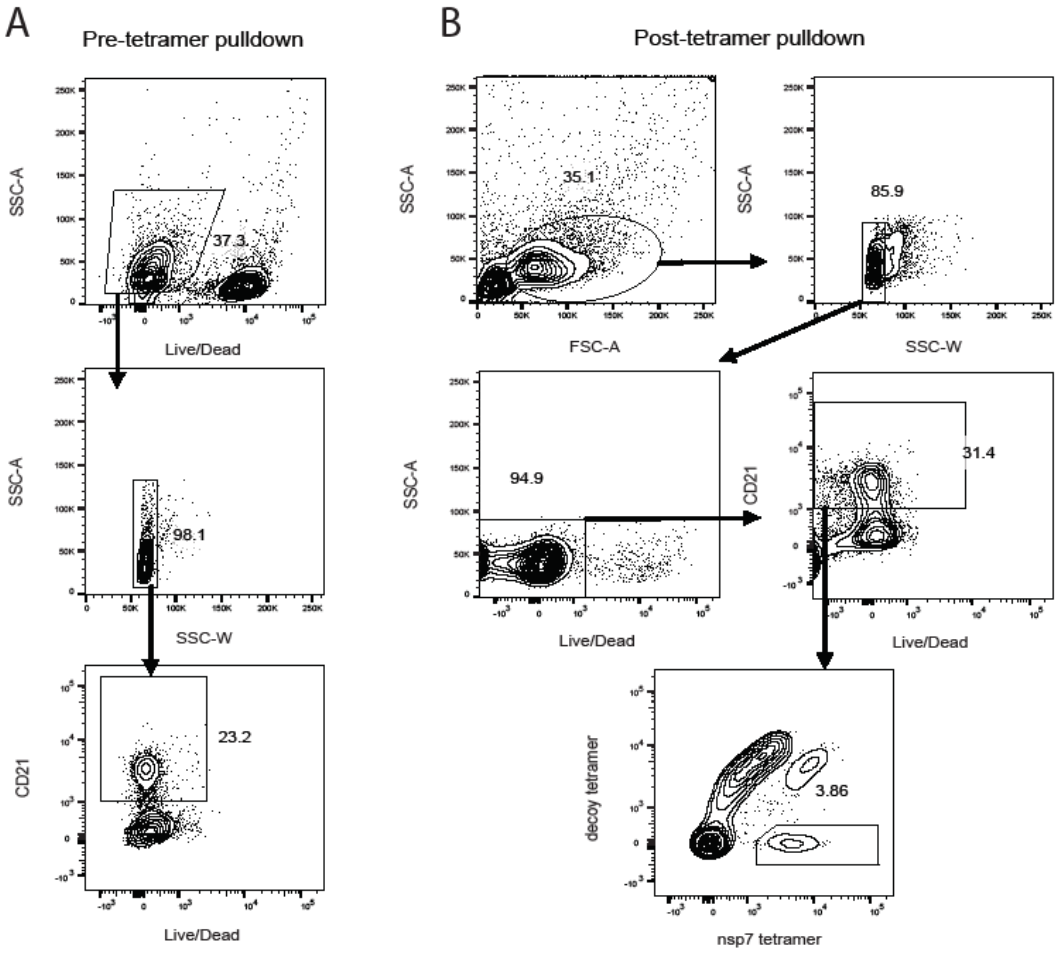

Figure 4-3 Flow cytometry gating example for nsp7-specific B cells per million B cells

Fig 4.3 – Flow cytometry gating example for nsp7-specific B cells per million B cells. (A) Pre-tetramer pulldown sample gating evaluated for live cells, single cells, and B cells. (B) Post-tetramer pulldown sample gating evaluated for lymphocytes, single cells, live cells, B cells, and nsp7 vs decoy tetramer.

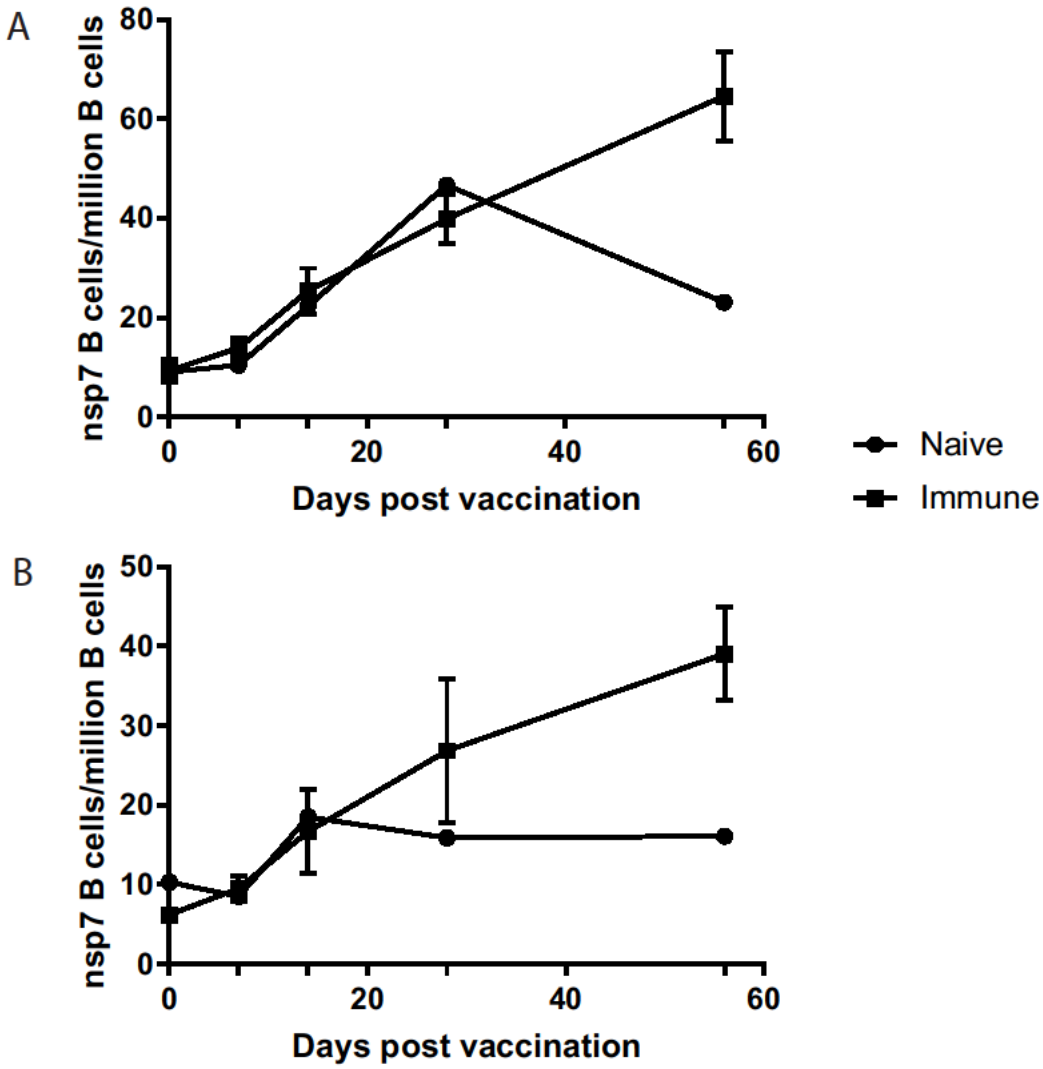

Figure 4-4 Kinetics of nsp7-specific B cell response post-vaccination for spleen and blood

Figure 4.4 – kinetics of nsp7-specific B cell response post-vaccination for spleen and blood. Each immune data point corresponds to mean +/- SEM with three animals per point. (A) nsp7-specific B cells per million B cells within PBMC population. (B) nsp7-specific B cells per million B cells within splenocytes.

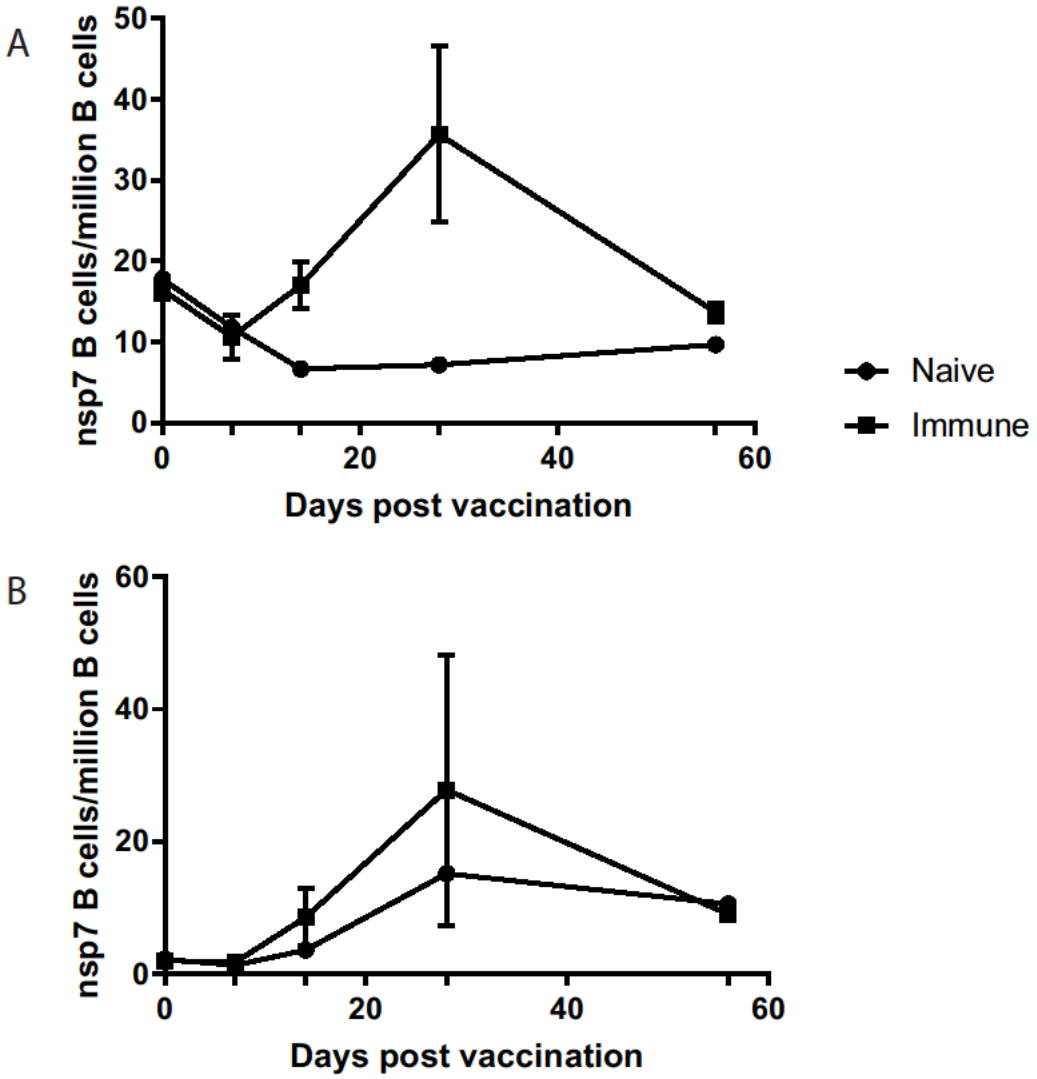

Figure 4-5 Kinetics of nsp7-specific B cell in the TBLN and tonsil

Figure 4.5 – kinetics of nsp7-specific B cell in the TBLN and tonsil. Each immune data point corresponds to mean +/- SEM with three animals per point. (A) nsp7-specific B cells per million B cells within the TBLN. (B) nsp7-specific B cells per million B cells within the tonsil.

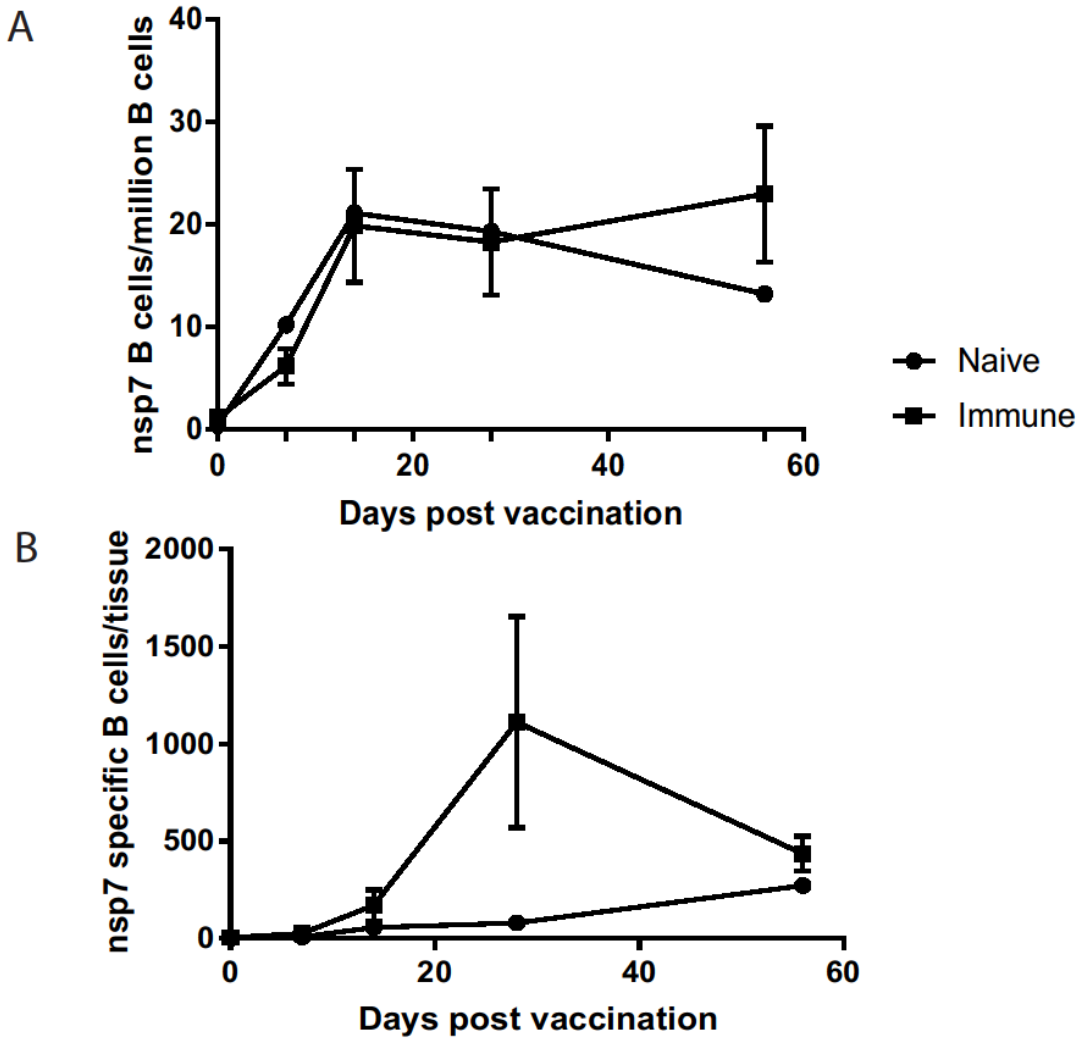

Figure 4-6 kinetics of nsp7-specific B cell in the superficial inguinal lymph node

Figure 4.6 - kinetics of nsp7-specific B cell in the superficial inguinal lymph node (ILN). Each immune data point corresponds to mean +/- SEM with three animals per point. (A) nsp7-specific B cells per million B cells within the ILN. (B) nsp7-specific B cells found upon analysis of entire lymph node for each animal.

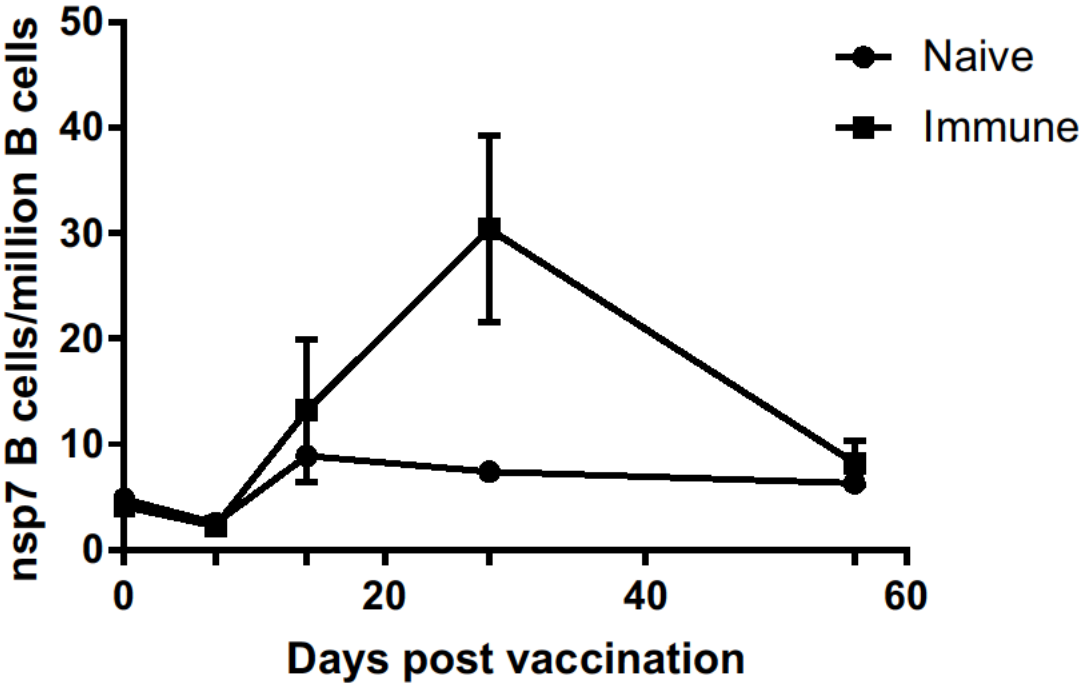

Figure 4-7 kinetics of nsp7-specific B cell in the mesenteric lymph node

Figure 4.7 - kinetics of nsp7-specific B cell in the mesenteric lymph node (MLN). Each immune data point corresponds to mean +/- SEM with three animals per point. Data are represented as the nsp7-specific B cells per million B cells within the MLN evaluated at 5 times points over the course of 56 days.

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Appendix 1: Establishment and characterization of a porcine B cell lymphoma cell line

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5.1 Abstract

The lack of well characterized, and established, domestic porcine cell lines hinders advancement of porcine cellular immunology understanding of disease resistance and anti-viral immunity. Numerous cases of multicentric lymphoma were diagnosed in pigs at the time of slaughter. Affected organs were harvested and submitted for single cell isolation and analysis. Culture of disaggregated single cell suspensions in RPMI 1640 with weekly passage resulted in clusters of dividing cells in about 2% of attempted isolations. In one pig, cells grew in approximately 50% of wells. Cell lines were established by limiting dilution repeated 3 times from splenic and subiliac lymph node lymphomas. Initial flow cytometry analysis showed a population of CD3-, CD79a+, CD21+, CD4-, and CD8- cells which have grown and been maintained in culture for more than 16 months up to passage number 60. To further characterize these rapidly proliferating cells, transcriptome analysis was carried out. This validated initial cytometric findings and confirmed that MIKE cells are B cells. This new porcine B cell lymphoma cell line will be a valuable resource for more in-depth cellular investigations into the porcine immune system, as well as providing a potential tool for the growth of lymphotropic viruses of pigs and humans.

5.2 Background and Introduction

The development of protective immune responses against many of the current pathogens of swine would benefit the health of swine as well as improve animal husbandry and production. These advancements in immunity are dependent upon an enhanced understanding of the porcine immune system, particularly at the cellular level. Currently, there is a dearth of reagents and cell lines with which to study porcine lymphocytes. This makes the development and characterization of new tools essential for the advancement of swine immunology and the improvement of animal welfare. Here, we describe the development of a new porcine B cell lymphoma cell line. While there have been many previously reported cases of swine lymphoma, this is the first case, to our knowledge, where a cell line has been created (Ogihara et al., 2012; Rafferty et al., 2007; Rocha et al., 2011).

A market weight pig, approximately 260 lb., was diagnosed with multiple grossly enlarged lymph nodes at time of slaughter. The carcass was immediately condemned. Sections from the spleen, subiliac and mesenteric lymph nodes were isolated, placed in tissue culture media, and sent overnight to the Murtaugh lab at the University of Minnesota. Upon receipt, the tissues were immediately broken down into single cell suspensions and cultured with daily observation for growth in cell numbers. In addition, the cells were phenotyped with flow cytometry, which identified the cancer as a B cell lymphoma. Several weeks post-culture, cellular proliferation was noted in many of the wells from all three tissues. Using multiple limiting dilution cultures, each time selecting for the highest dilution with growing cells where the next lowest dilution did not show growth, the cells were cloned out until the three fastest growing clones were isolated.

RNA sequencing was performed to identify differences in key gene expression as well as to rule out an RNA virus as the causative agent of the lymphoma.

5.3 Materials and Methods

Cellular isolation and culture

Spleen and lymph nodes were collected from market weight pigs displaying signs consistent with lymphoma. Tissues were sectioned into 1 cm x 1 cm x 1 cm cubes. Three cubes of tissue were placed into 50 ml conical tubes of complete RPMI with 5% FBS, and then shipped overnight on ice. Tissues were received the next day. Dissociation of cells from surrounding structural tissue was achieved through forceful pressing of isolated tissue on a metal screen into culture media within a petri dish. Cellular suspensions were then aspirated and passed over a 40 µm filter into fresh 50 ml conical tubes. Following centrifugation at 1400 rpm for 10 minutes, the supernatant was decanted, and the pellet was resuspended in ACK lysis buffer (Lonza) and then incubated for 10 minutes. Cells were washed with complete RPMI and then centrifuged again. Cells were resuspended in 10% RPMI, counted, and then plated at 50,000 live cells/well of a 96 well plate. Remaining cells were put into freezing media (50% FBS, 40% Complete RPMI, 10% DMSO) at ~10 million cells/ml and frozen in liquid nitrogen following several cooling steps. Cells were visualized every day for the presence of proliferation. Media was changed every 5 days and cells were stained with trypan blue, counted and evaluated for viability.

Cell Cloning

Culture wells in which cellular proliferation was identified were removed from culture, counted for live cells, and then re-cultured by ten-fold dilutions starting with 100,000 cells working down to 10 cells. Cells were left in culture until obvious proliferation was noted. The lowest dilution well which displayed proliferation was selected, grown up, and then re-cultured by ten-fold dilutions as previously described. This was performed three times after which the four fastest proliferating clones from lymphoma cells isolated from the iliac lymph node and two fastest proliferating clones from the spleen were selected for RNA extraction.

RNA extraction and sequencing

Four lymphoma cell clones isolated from the iliac lymph node and two lymphoma cell clones isolated from the spleen were selected for RNA sequencing and analysis due to their superior proliferating capabilities. Splenocytes from a healthy ~115 kg female pig were magnetically enriched for CD3 (T cells) and CD21 (B cells) expression for healthy lymphocyte controls. Lymphoma cell clones and magnetically enriched control cells were counted and normalized. The protocol from a Qiagen RNeasy, RNA extraction, kit was followed for all samples. Isolated total RNA was submitted to the University of Minnesota Genomics Center for quality control analysis and finally paired end read sequencing on an Illumina HiSeq 2500.

Flow cytometry

Four color flow cytometry was performed using an LSRII (BD Biosciences) with commercially available reagents on primary tumor cell homogenates and cultured cell subclones. The following antibodies were used: unlabeled mouse anti-human CD79a mAb (abcam HM47/A9), FITC mouse anti-porcine CD3 mAb (abcam PPT3), phycoerythrin (PE) mouse anti-porcine CD21 mAb (Southern Biotech BB6-11C9.6), fixable viability dye eFluor780 (eBioscience). Unlabeled anti-CD79a antibody was conjugated to APC using an abcam APC antibody conjugation kit.

Seahorse assay analysis

An XF24 tissue culture plate (Agilent) was treated for 30 minutes with 1 ug/well of Cell-tak (Corning). The plate was then washed with sterile water and kept overnight at 4C. An XF24 sensor cartridge was hydrated with XF calibrant solution and incubated overnight at 34 C. On the day of the experiment, splenocytes from an apparently healthy adult female pig were thawed from liquid nitrogen and then enriched for CD21 expression using magnetic enrichment (MACS Miltenyi Biotec). Cell lines were removed from culture and all cells were counted using a hemocytometer. All cells were centrifuged at 1400 RPM for 5 minutes, culture media was aspirated, cells were washed in unbuffered DMEM, and then plated at 300,000 live cells/well. Cells were incubated in unbuffered DMEM for 1 h at 37 C prior to assessing oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in the Seahorse XF-24 analyzer.

Cell energy phenotype was assessed using a Seahorse XFp cell energy phenotype test kit (Agilent). Oligomycin (1 uM/well) and FCCP (.5 uM/well) concentrations were optimized and then utilized for the assessment of ATP production and maximal respiration capacity. Total experimental time was approximately 90 minutes for each plate.

5.4 Results and Discussion

Establishment of the cell lines

Primary cells were obtained from an adult pig diagnosed with multicentric lymphoma at slaughter. The lymph nodes and spleen of the animal were grossly enlarged (Fig 5.1). The animal's carcass was immediately condemned and a full necropsy was performed by a veterinarian. The lymph nodes, as well as the spleen, were sectioned, placed in containers with complete RPMI and then shipped overnight for cellular isolation and culture. Cells were cultured at 50,000 live cells/well in 96 well round bottom plates and observed daily for marked changes in cell number or conformation. At four weeks post-culture it was noted that several wells, with cells from the both the subiliac lymph node and spleen, had large populations of proliferating cells. Cells from proliferative wells were removed and re-cultured in tenfold limiting dilutions for cellular cloning. Two lymphoma clones from the spleen and four from the subiliac lymph node were selected for proliferative capacity. Following passage 15, the clones were frozen in liquid nitrogen for a period of one week and then thawed and re-cultured. Following passage of thawed cells, one clone from the spleen and two clones from the subiliac lymph node were selected, due to their rapid

proliferation, and named MIKE001-MIKE003 respectively. Clones were passed 1:10 every six days with an average doubling time of approximately every 16 hours.

Cellular characterization

Initial flow cytometric analysis characterized all MIKE cell clones as B cell lymphomas based on $CD21^+$, $CD79a^+$, and $CD3^-$ extracellular protein expression (Fig 5.2). IHC staining confirmed this, as well as identifying MIKE cells as $CD20^+$ and $PAX\text{-}5^+$ (data not shown). Subsequent RNA sequencing was carried out on MIKE001-003, as well as CD3 or CD21 magnetically enriched healthy splenocytes from an age matched pig. Analysis of FPKMs (fragments per kilobase of transcript per million mapped reads) via ingenuity pathway analysis (IPA) identified the down expression of p53 in the lymphoma cell lines compared to healthy lymphocytes (Fig 5.3). This was determined to be a key difference in gene expression leading to a change in the expression of downstream genes such as MYC, RB1, and cdkn1. The change in regulation of the expression of these genes is likely responsible for the unabated proliferation displayed by the MIKE cells.

Unmapped RNA sequencing reads were analyzed with Kraken to identify the presence of RNA viruses which may have been the etiologic agent of the lymphoma. Kraken identified one retrovirus in all samples, MIKE cells and controls, which was determined to be part of the endogenous porcine AKT1 gene. Therefore, a causative agent of this lymphoma could not be identified in this study.

Cellular metabolism

Initial tracking of proliferation curves showed that MIKE003 cells grew slightly slower than MIKE001-2 (Fig 5.4A). As a result of this, we hypothesized that there was a difference in metabolism between MIKE001-2 and MIKE003. A seahorse xf analyzer was utilized to measure cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Comparison of these values between our MIKE cells and CD21+ positive enriched primary B cells showed that MIKE001 cells are the most metabolically active cell clone (Fig 5.4B). MIKE002 cells are more active than MIKE003 cells but not as much as MIKE001. While MIKE001 cells are more metabolically active than primary cells they are considerably less active than the other B cell lymphoma clones. These data corresponded well with the proliferation curves.

Conclusions

Here, we described the isolation, development, and characterization of a porcine B cell lymphoma cell line. MIKE cells are CD21+, CD79a+, CD3- non-adherent cells which grow rapidly in cell culture and have been passed up to 60 times. These cells are another tool by which the porcine immune response may be studied. Furthermore, they may be important for the growth of lymphotropic viruses of both animals and humans.

5.4 Acknowledgements

We thank Juanito Abrahante Llorens and the University of Minnesota Informatics Institute for their assistance with RNA sequence analysis. The research was supported by

USDA NIFA grant 2015-06966 and an NIH fellowship to MCR from the training grant T32 OD010993.

Figure 5-1 Comparison of market weight spleen size

Figure 5.1 – Comparison of market weight spleen size. (A) Grossly enlarged spleen next to 18 inch metal ruler. (B) Apparently healthy spleen next to 18 inch ruler.

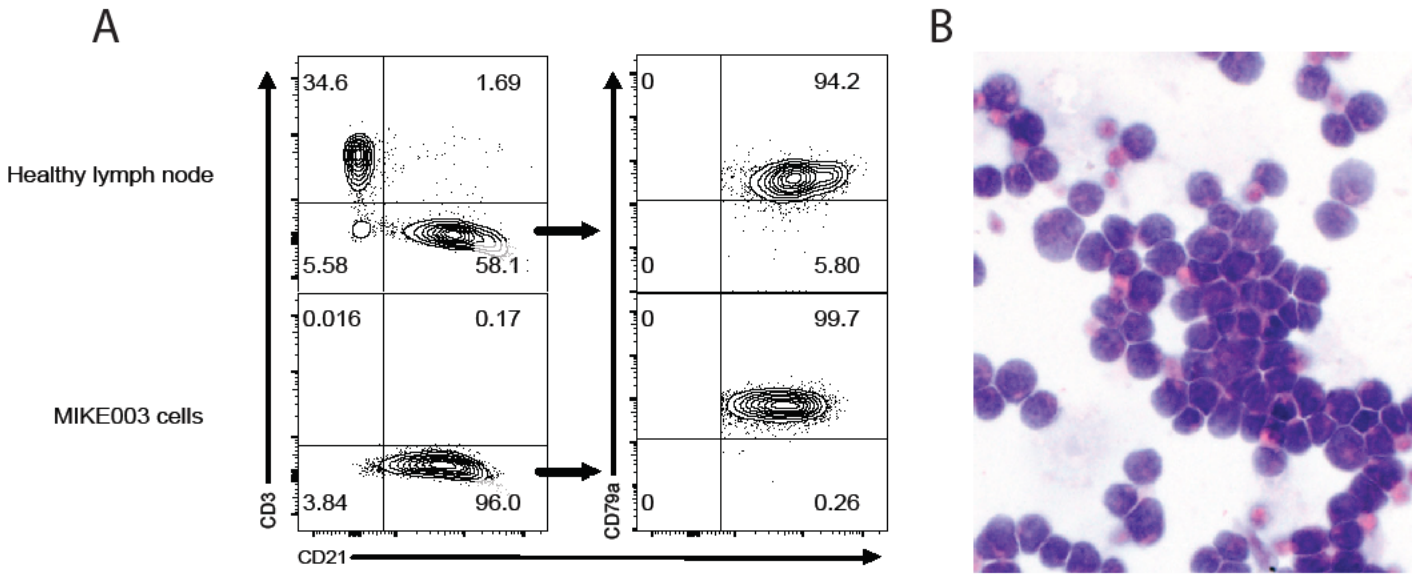

Figure 5-2 Cell line characterization

Figure 5.2 – Cell line characterization. (A) Flow cytometry of MIKE003 cells compared to cells from an apparently health subiliac lymph node from a market weight pig. (B) H&E staining of MIKE003 cells (40x objective).

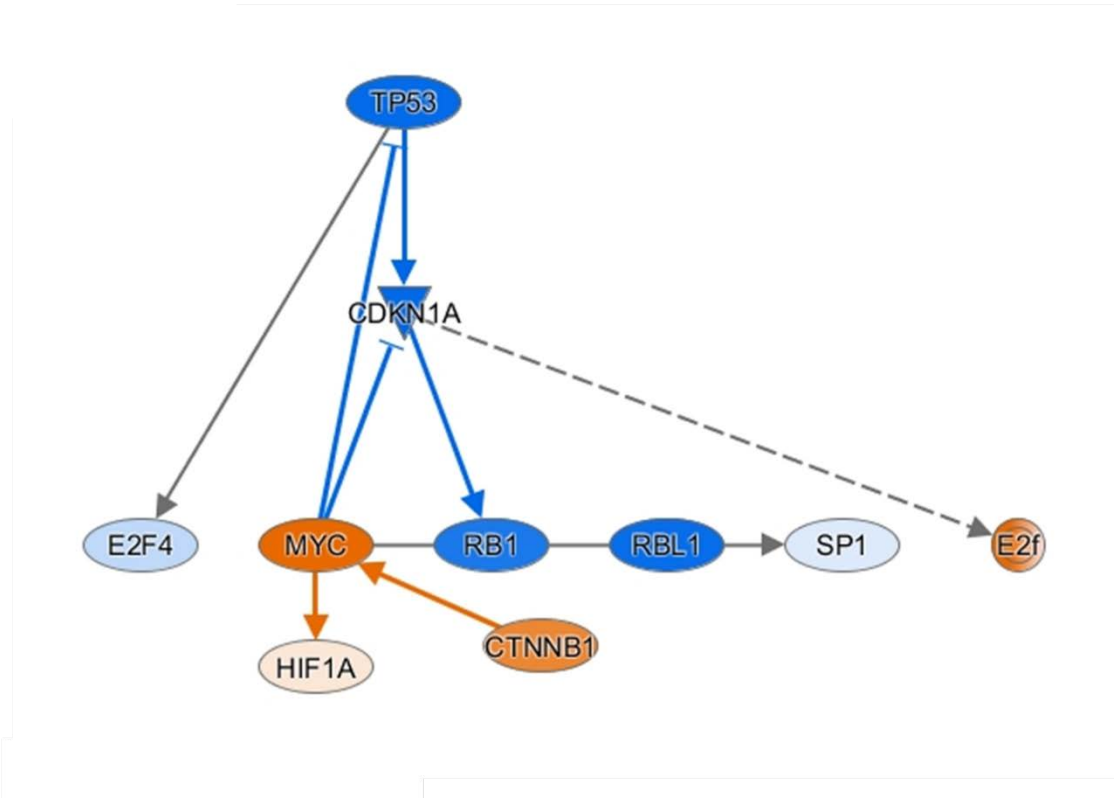

Figure 5-3 Schematic diagram of pathway regulation

Figure 5.3 – Schematic diagram illustrating differences in pathway regulation in MIKE cells compared to healthy lymphocytes with IPA analysis.

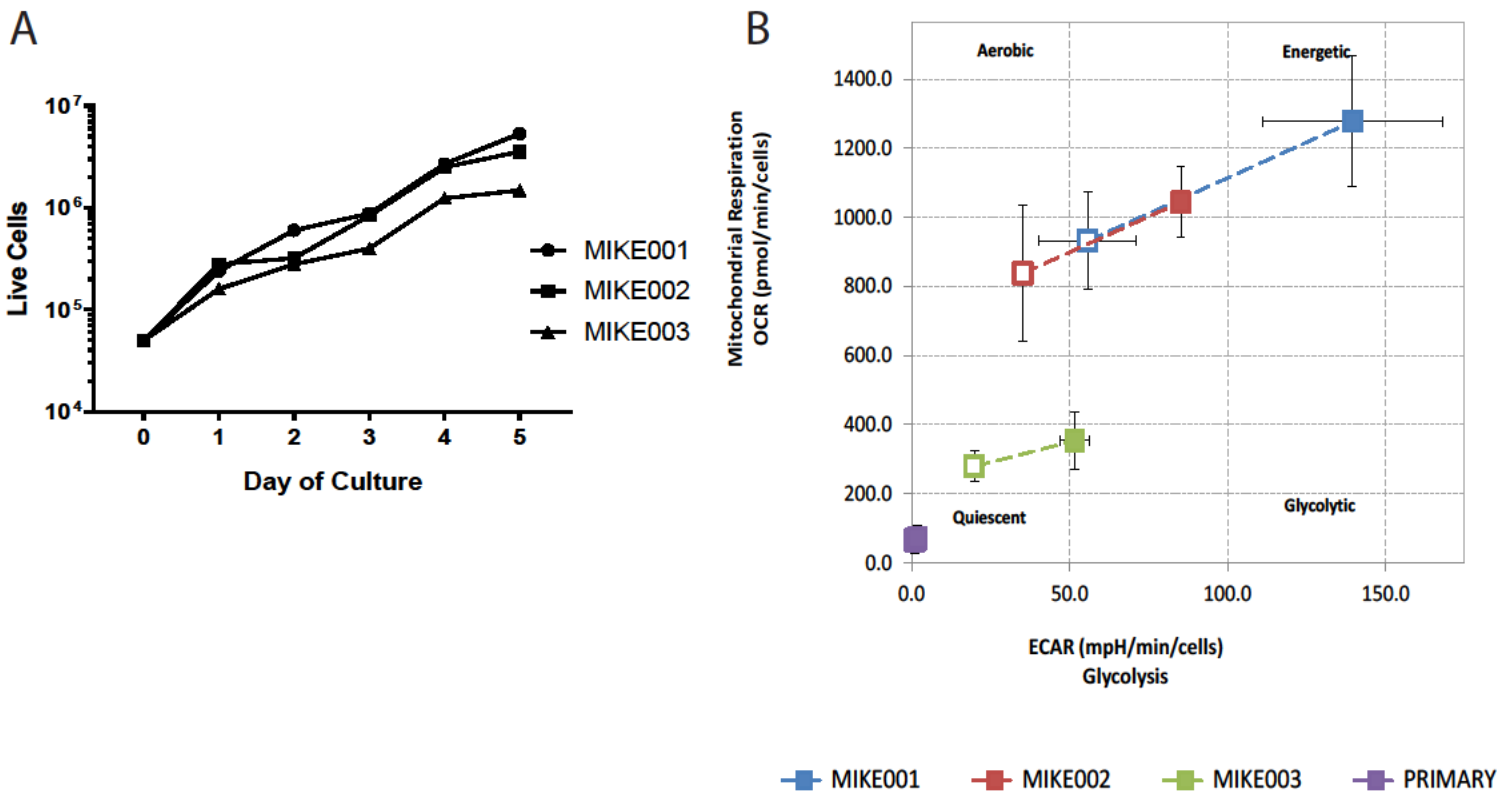

Figure 5-4 Cell proliferation and metabolism

Figure 5.4 – Cell line proliferation and metabolism. (A) Cell lines were evaluated for cellular proliferation each for five days with a hemocytometer. (B) Seahorse analysis of cellular metabolism for each of the three cell lines.